Modelling Pseudomonad Growth in Milk and Milk-based Products

Karina Neumeyer B.Sc. (Hons.)

submitted in fulfilment of the requirements for the degree of
Master of Science
University of Tasmania
Hobart
November, 1995

DECLARATION

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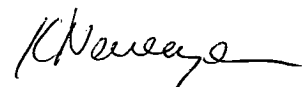

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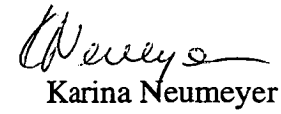

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ABSTRACT

Predictive microbiology is a method by which the growth responses of microorganisms of concern are modelled mathematically in respect of the major factors affecting growth. A model for the growth of *Pseudomonas* (the main organism of concern in refrigerated milk and some milk products) was developed and validated.

Psychrotrophic psuedomonads were isolated from various modified and whole milks, using both Victorian (obtained from CSIRO Dairy Research Laboratories) and Tasmanian milks. Growth rates in artificial broth media were determined using turbidimetric methods. By fitting a sigmoidal curve (using the Gompertz function) to the data, generation times can be calculated and growth rates (by turbidimetric or viable count methods) determined. All strains were grown in artificial broth media at 10°C and growth rates compared. The fastest growing strain (*Pseudomonas putida* 1442) was used to develop the model i.e., a worst case approach was adopted. A square root model (Ratkowsky *et al*, 1982) was developed for *P.putida* 1442 in artificial broth media by determining the generation time at 0.5°C intervals from 0 to 50 °C. A similar procedure was used to model the effect of water activity (using sodium chloride as the solute) on *Pseudomonas* spp.

Generation times calculated by turbidimetric and viable counts were found to differ. This difference was found to be constant with respect to temperature and was incorporated into the modelling process so that all models expressed generation times equivalent to those calculated by viable counts, the standard method for enumerating microorganisms in food products.

A literature search comparing the notional minimum temperature (T_{min}) for growth of psychrotrophic pseudomonads found that the T_{min} was the same (265.4K ± 0.7) despite the source of the organism. This implies that only one model for temperature dependence is needed for psychrotrophic*Pseudomonas* in dairy, meat and poultry products.

The model was validated both in the laboratory and in industry using various milk and milk based products. The validation process involved monitoring the growth of pseudomonads at various temperatures and comparing the observed generation times to those predicted using bias and accuracy factors (Ross, 1993). The temperature model has been incorporated into prototype computer software and

trialled in the dairy industry. The validation process showed the psychrotrophic pseudmonad model to accurately predict the growth of pseudomonads in the products tested. In some instances in industry, further information in the form of the duration of lag phase, is required to maximise the accuracy of the model. The capacity to input this information was included in the prototype software.

ACKNOWLEDGMENTS

I would like to thank my supervisors Prof. T.A. McMeekin and Dr. J. Olley for providing me with the opportunity to undertake this research and for their support and constructive criticism throughout this study.

I would also like to thank;

- The various funding bodies that provided financial support; these being the Rural Industries Research and Development Corporation, Dairy Research and Development Corporation, Meat Research Corporation and Pig Research and Development Corporation.

- Dr. Tom Ross for his support, encouragement, practical advice and criticisms

- Dr. David Ratkowsky for freely sharing his statistical knowledge

- Simon James for his computer assistance

- Amabel Fulton for sharing her knowledge on questionnaire techniques

- all the the staff and postgraduate students in the Department of Agricultural Science for the help, good ideas and all the fun times.

- Most importantly I'd like to thank my husband, Martin, for his understanding and unlimited support, without which, this work would not have been possible.

ABBREVIATIONS USED IN THIS THESIS

a_w	water activity
$a_{w\ min}$	notional minimum water activity for growth
API	Analytical Profile Index
CFU	colony forming unit
CI	confidence interval
GT	generation time
LPD	lag phase duration
MAFF	Ministry of Agriculture Food and Fisheries (UK)
MPD	maximum population density
n	number of growth curves
pH_{min}	notional minimum pH for growth
PSA	Pseudomonas Selective Agar
PCA	Plate Count Agar
r^2	regression coefficient squared
$\sqrt{r}$	$\sqrt{}$growth rate
SD	standard deviation
%T	% Transmittance
TGI	Temperature Gradient Incubator
T_{min}	notional minimum temperature for growth
T_{max}	notional maximum temperature for growth
T_{opt}	optimum temperature for growth
VC	Viable Counts
the model	refers to the model for *P. putida* 1442 (Equation 4.3)

PUBLICATIONS

Refereed Publications

Dalgaard, P. Ross, T., Kamperman, L., Neumeyer, K. and McMeekin, T.A. (1994) Estimation of bacterial growth rates from turbidimetric and viable count data. *International Journal of Food Microbiology*, **23**: 391 - 404.

Other Publications

Ross, T., Neumeyer, K., Kamperman, L. and McMeekin, T. (1993) In defence of predictive microbiology. The Australian Microbiologist. 14: 103 - 107

Computer Software Package

Ross, T., Ridge, J. & Neumeyer, K. (1993) *Pseudomonas* Predictor. Computer software package available in Lotus 123 and Microsoft Excel with Users Manual.

Conference Posters

Neumeyer, K., Ross, T., & McMeekin, T.A. (1995) Has Your Product Spoiled?. 8th AIFST Conference. Victoria. Australia.

Neumeyer, K. and Kamperman, L. (1993) Predicting the growth of psychrotrophs in milk and meat. Annual Scientific meeting of the Australian Society for Microbiology. Perth. Australia.

The microbe is nothing; the terrain everything

- Louis Pasteur

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1. LITERATURE REVIEW

1.1. MICROBIOLOGY OF DAIRY PRODUCTS

1.1.1. Milk

In 1992/3 Australia had over 1.6 million dairy cows producing some 7.33 billion litres of milk. Due to pasture grazing, as opposed to the use of feed-lots, milk production in Australia generally follows seasonal trends with the majority of milk being produced in October and November. Milk production is directed into two areas: market milk for direct consumption (25%) or manufacturing milk which is converted into various dairy products (75%) (Prattley, 1995; Sullivan, 1994). Australia accounts for almost 10% of the world dairy trade and in 1992/3 around 35% of the total national dairy production, valued at over $1 billion, was exported (Sullivan, 1994).

Temperature, water activity and pH are often considered to be the three most important factors that influence the rate of growth of microorganisms (Roberts & Jarvis, 1983; Farber, 1986; McMeekin & Ross, 1993). Milk is a well balanced medium, containing carbohydrates (47 g/L), fats (36 g/L), protein (33 g/L), minerals and vitamins (Marshall, 1992). In products, such as liquid milk, where pH, a_w and nutrients are optimal for growth, the only significant factor affecting the growth rate of bacteria is the storage temperature (McMeekin & Olley, 1986). Prior to compulsory pasteurisation, milk was one of the major vehicles for transmissible disease e.g., typhoid fever, scarlet fever, tuberculosis, polio, shigella, hepatitis A, diphtheria and brucellosis, and yet when pasteurisation was introduced into the USA, in 1895, many milk processers resorted to secretly pasteurising the milk due to public concern regarding perceived negative effects of the process on the milk (Klein, 1917). Other improvements in milk quality are due to the elimination of some diseases in dairy cattle, improved sanitation on farms and in factories and the education of producers, processors and consumers (Richardson, 1985).

1.1.1.1. Contamination of Milk

The number of bacteria contaminating milk at the farm level varies from 10^3 to 10^6/mL (Law & Mabbitt, 1983). Contamination may occur from a number of sources including the interior of the udder, the cows teats and the milking and storage equipment (Cousin, 1982; Chapman & Sharpe, 1983; Tatini *et al*, 1991). The interior of the udder contributes about 2% of contamination and is usually only of concern if the cow is suffering from infection e.g., mastitis.

The number of bacteria in raw milk affect not only the shelf life and sensory properties of the milk but also result in the deterioration of milk quality and product yield due to the production of heat tolerant lipases (which cause rancid defects in cheese) and proteases (which degrades the casein causing UHT milk to gel, productions of bitter flavour defects and decreased yields). These extracellular enzymes are often produced by psychrotrophic contaminants prior to pasteurisation (Adams *et al*, 1975; Law, 1979; Chapman & Sharpe, 1983; Tatini *et al*, 1991; Marshall, 1992) and are usually due to *Pseudomonas* species, in particular *P. fluorescens, P. putida* and *P. fragi* (Law & Mabbit, 1983; Shelley *et al*, 1986, 1987). The number of Gram negative bacteria may be very low initially. However, due to their ability to grow at refrigeration temperatures they (in particular the pseudomonads) often play one of the most important roles in milk spoilage (Shelley *et al*, 1987; Tatini *et al*, 1991). The percentage of pseudomonads in raw milk can vary from 20 - 80% of the total biota depending on the cleanliness of the establishment (the better the hygiene the fewer pseudomonads found).

Pseudomonads are also the major cause of spoilage in pasteurised milk (Hussong *et al*, 1937; Chandler & McMeekin, 1985a,b; Griffiths *et al*, 1988; Chandler *et al*, 1990). Craven *et al* (1994) found that 86.8% of microorganisms in milk at spoilage belonged to the genus *Pseudomonas*. It must be assumed that these pseudomonads are post-pasteurisation contaminants as they cannot survive the pasteurisation process (Cousin, 1982). At temperatures above 15°C, mesophilic organisms may start to become dominant (Chandler & McMeekin, 1985b; Griffiths & Phillips, 1988a,b) although this is dependent on the initial levels of contamination of the pseudomonads and other organisms.

1.1.1.2. Detecting Psychrotrophic Bacteria

The current standard method for detecting psychrotrophic bacteria in milk involves incubating agar plates at 7°C for 10 days (Cousin, 1982; Tatini *et al*, 1991), however, it is often impractical to hold perishable foods, such as milk, while waiting for test results. There are many methods (e.g., calorimetry, impedance, gas-liquid chromatography to detect metabolites, estimation of acetaldehyde in milk headspace (Cousin, 1982)) that can be used to indicate the levels of contamination. However, they are often time-consuming, expensive, lack reproducibility or simply do not correlate well with actual numbers of bacteria in the milk. Dye reduction tests, such as methylene blue or resazurin, and the direct microscopic count (DMC) remain the current methods of providing relatively fast ($\approx$ 30 minutes), cheap and reproducible counts, although bacteria of no significance will be included in the count (Chandler & McMeekin, 1985b; Adams & Moss, 1995). Another test is the

direct epifluorescent filter technique (DEFT) in which a sample is filtered through a membrane filter to capture bacteria, dyed with a fluorescent dye and examined under a microscope. DEFT has the advantage of being rapid (25-30 minutes), sensitive and can differentiate viable and non-viable cells (Sharpe, 1994). As with the dye reduction tests and DMCs, however, it does not differentiate total counts and psychrotrophic pseudomonads. A more recent method developed by Craven *et al* (1994) uses an MPN technique for detecting gram-negative bacteria in milk in which the milk is mixed with a selective agent (benzalkonium chloride) and a bacterial growth indicator (tetrazolium salt). The benzalkonium chloride suppresses growth of gram-positive organisms while the tetrazolium salt results in a change of colour of the milk from white to pink once gram-negative organisms reach 10^7 per mL. The difficulties encountered with this test are those inherent for any MPN technique.

1.1.1.3. Methods of Controlling Pseudomonads

1.1.1.3.1. At the Farm

The first step in controlling pseudomonads is to reduce the initial microbial load, however many methods (e.g., washing the teats with NaOCl, followed by drying the teat) are impractical for the farmers or are not cost-effective (Law & Mabbitt, 1983). The main approach has, therefore, been to refrigerate the milk at the farm, thereby restricting the growth of bacteria and improving the quality of the raw milk. Increased attention to refrigeration results in better quality milk, but the cost (both in capital outlay and energy requirements) can become prohibitive. The use of predictive models would allow calculations to be made regarding whether the benefit of a decreased microbial load is worth the cost of implementing a new refrigeration scheme. Cost/benefit analyses will become increasingly important as more and more farmers work on a "quality-pays" system in which bonus payments are received for better quality milk. However the company must have in place extension officers available to help both milk producers and researchers collaborate and solve problems as they arise.

1.1.1.3.2. Activation of Inhibitory Systems

Bovine milk contains natural inhibitory compounds such as lactoperoxidase. The lactoperoxidase system contains three components; lactoperoxidase, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). All three are required for the antimicrobial effects (to which Gram negative organisms are very sensitive). The varying concentrations of the compounds are shown in Table 1.1.

Table 1.1 Concentrations of antimicrobial compounds in bovine milk and the amounts required for the antimicrobial effects to take place. (Jay, 1992)

compound	concentration required to be inhibitory	concentration present in bovine milk
lactoperoxidase	0.5 - 1 ppm	30 ppm
thiocyanate	0.25 mM	0.2 - 0.25 mM
hydrogen peroxide	100 μ/mL	1 - 2 μ/mL

Björck *et al* (1975), Reiter (1981) and Cousin (1982) suggest that it is the current more controlled bovine feeding regimes that are responsible for the low amounts of SCN^- naturally present in the milk. Cows fed on 'weeds' produce SCN^- as a side product of detoxification of cyanide (in clover, millet, peas, cassava, etc) and hydrolysis of glucosides by rhodanase (in crucifers such as kale, turnips, cauliflower and rape). Thus, in modern bovine diets where a large proportion of the feed comes from silage, feed concentrates, hay or ley pastures (usually containing very few grass species), the amount of SCN^- occurring naturally in the milk is very low. H_2O_2 is a metabolite of leucocytes and the level of H_2O_2 is thus dependent on the health of the udder (Reiter, 1981) although under normal conditions any H_2O_2 formed is rapidly reduced by catalase (Reiter & Härnulv, 1984).

The theoretical rate limiting step in activating the lactoperoxidase system is usually considered to be the amount of H_2O_2 naturally present in the milk, although, in practice, the levels of SCN^- may also be limiting. H_2O_2 can be added directly, produced by lactic acid bacteria or generated by glucose oxidase enzymes (Cousin, 1982; Law & Mabbit, 1983). One criticism of using this method is that it imparts a 'chemical like' flavour to cheese (Chapman & Sharpe, 1983) although Scott (1981) suggests that the addition of catalase will remove the residual H_2O_2. Cousin (1982) comments that cheese made from untreated milks became rancid within 4 months, while cheese made from treated milk still had normal flavour. However, these criticisms are valid if only H_2O_2 is added and not both H_2O_2 and SCN^-. Provided both substances are present in equimolar concentrations, the H_2O_2 is used up and disappears from the milk (Reiter, 1981).

Reiter (1981) found that given a constant non-inhibitory level of H_2O_2 the level of SCN^- can provide either a bacteriostatic (low levels of SCN^-)or a bactericidal (high levels of SCN^-) effect. The effect is temporary and once it ceases, growth of the microorganism continues at an exponential rate (Reiter, 1981). The lactoperoxidase system is used in developing countries, such as Kenya and Sri Lanka, where refrigeration is uncommon (Korhonen, 1980; Reiter, 1981).

1.1.2. Cream

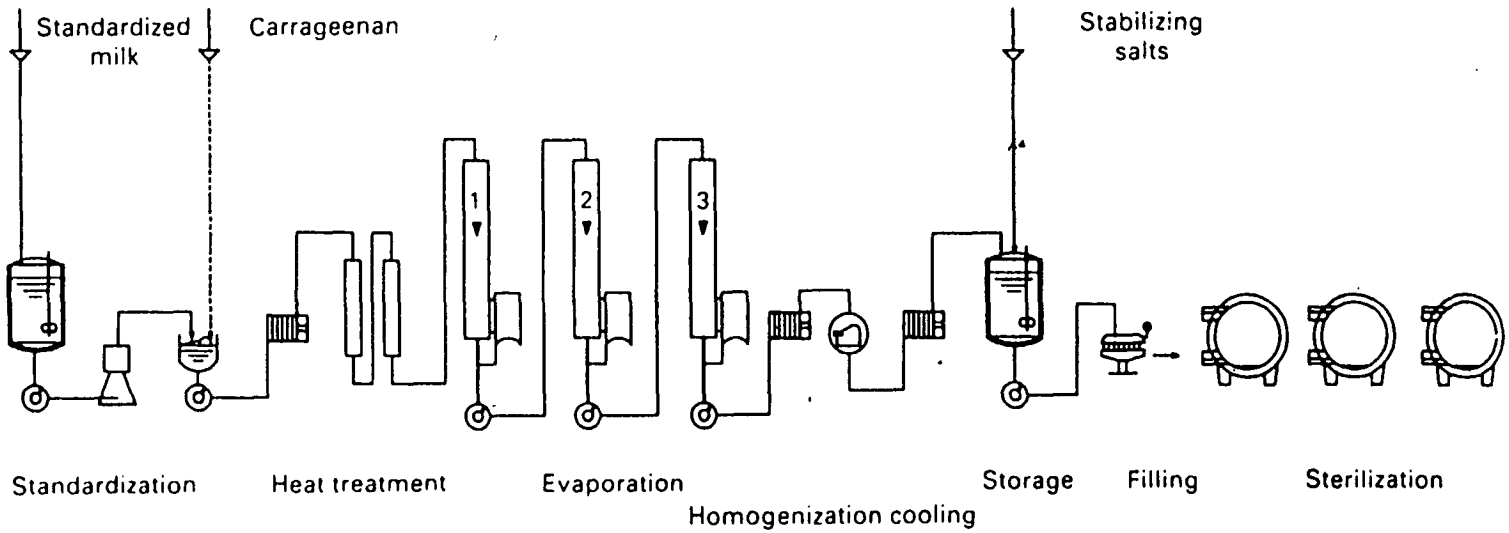
Cream is separated from milk by centrifugation and contains all the fat and a proportion of the protein and lactose in milk (Gaman & Sherrington, 1978). Typical additives, allowed in some countries, include sugar, emulsifiers, stabilisers and stabilising salts to produce a smooth product without graininess and to inhibit the agglomeration of fat globules. Cream is usually pasteurised at 80°C (compared to 72°C for milk), however, increasing the temperature to 85°C can shorten the shelf life possibly due to the activation of bacterial spores (Towler & Cant, 1993).

The biota of cream is very similar to that of whole milk, since as the fat droplets rise to the surface, they carry up the microorganisms (Jay, 1992; Adams & Moss, 1995). Cream is classified according to the fat content and can range from extra light cream (12% fat) to rich cream (55% fat) and may include both treated (e.g., cultured sour cream, clotted cream) and natural creams (Prattley, 1995).

1.1.3. Evaporated Milk

Evaporated milk is made by reducing the water content of whole milk by about 60% through an evaporation process (Jay, 1992). Legislation in some countries allows the addition of carrageenan, up to 150ppm, to improve phase stability (Hess, 1993). The milk is then heat treated, sprayed from the top of a series of vertical tubes and dispersed by a spreader plate. The milk forms a thin film on the inside of the tubes and falls down the walls. Water is evaporated from the product and steam removed as a condensate. Stabilising salts, such as sodium, potassium and calcium salts of hydrocholoric acid, citric acid, carbonic acid, orthophosphoric acid or polyphosphoric acid, may be added, to the homogenised evaporated milk, prior to canning. Evaporated milk is sterilised to commercial sterility by conventional retorting or aseptic processing. A flow diagram for the manufacture of evaporated milk is shown in Figure 1.1.

Figure 1.1 A basic flow diagram for the manufacture of evaporated milk



(Hess, 1993)

The evaporation process has the effect of increasing the total solids from 12% to 22 and 28% for reduced fat and whole milk respectively (Prattley, 1995). The removal of water results in a reduced water activity and a long shelf life milk product. When premature spoilage of evaporated milk occurs, it can usually be traced back to insufficient can integrity arising from faulty soldering, welding of cans or closure seams, although excessive amounts of thermophilic spores may also cause spoilage (Hess, 1993).

1.1.4. Cottage Cheese

Cottage cheese is a soft unripened cheese made from skim milk to which cream and salt may be added. Cottage cheese with pH <5.0 has the longest shelf life while those with pH values up to 5.2 - 5.3 have a minimal keeping quality and a blander flavour (Kosikowski, 1970).

The primary bacterial contaminants of cottage cheese are *Alcaligenes*, *Pseudomonas, Proteus,* and *Aeromonas*, (which cause off-flavours, pigment formation and slimy curd) and yeasts and moulds e.g.,*Geotrichum*, *Alternaria, Penicillium* and *Mucor* (which impart stale, musty, mouldy and yeasty flavours) (Jay, 1992; Chen & Hotchkiss, 1993).

The starter culture for cottage cheese is *Streptococcus cremoris*, or *S. lactis* subsp *diacetylactis* (now reclassified as *Lactococcus lactis* subsp *cremoris* and *L. lactis* subsp *lactis* respectively) as the dominant acid producers and *Leuconostoc cremoris* or *L. citrororum* as the dominant flavour producers (Chapman & Sharpe, 1983; Green, 1984; Kosikowski, 1970). The digestive enzyme rennin (derived from stomachs of suckling calves, lambs and goats) is used to coagulate skim milk, which is then cooked to achieve a moisture content of 80% or less, a water activity of 0.99 (Rowe, 1993) and washed to cool the curd and remove the whey. Contamination often occurs during the setting period or from the chilled water used for rinsing the curd. Contaminants from the setting period are usually *Aerobacter* (now called *Enterobacter*) and lactose fermenting yeasts, resulting in gassiness in the curd, while those from the chilled water are usually psychrotrophs such as *P. fragi* and *P. viscosum* which convert the diacetyl to acetylmethylcarbinol resulting in a lack of aroma (Foster *et al*, 1958; Chapman & Sharpe, 1983).

1.2. INTRODUCTION TO PREDICTIVE MICROBIOLOGY

In the dairy industry, milk must be processed quickly in order to reach the consumer or processor as fresh as possible. Preservatives and other additives cannot be used due to the 'whole, fresh' image that advertisements project for milk and that consumers are consequently demanding. Many of the faster chemical methods for determining the numbers of bacteria are expensive, often depend on detecting spoilage metabolites, such as sulphides and amines, and cannot detect numbers of bacteria below 10^{7-8} cells/g at which point the product is already at incipient spoilage (McMeekin & Olley, 1986; McMeekin & Ross, 1993). As a result current methods of detecting spoilage are diagnostic rather than predictive; that is, they may show that spoilage is incipient but provide no indication of remaining shelf life. Diagnostic methods of detecting spoilage also tend to be destructive and so limited amounts of product, compared to the total produced, can be tested. Producers therefore need to ensure quality management within the food manufacturing, distribution and retailing chain.

In the past, if a product was reformulated, it was necessary to repeat the storage trials and microbial challenge tests to ensure that the safety of the product was not compromised. The papers by Griffiths *et al* (1987) and Hart *et al* (1991) are representative of many papers published. Griffiths *et al* (1987) studied the effect of two temperatures on the growth of the natural biota of milk, while Hart *et al* (1991) studied the effect of three temperatures and four gaseous environments on the growth of *Listeria monocytogenes* on chicken breasts. While experiments of this nature are useful for the product in question, the results cannot be extrapolated to any other products or situations and any change to the formulation or conditions would require that the challenge tests be repeated. For example, Griffiths *et al* (1987) showed that the shelf life of raw milk at 2°C is longer than at 6°C by a factor of 1.8 and that thermisation at 65°C for 15 seconds increases shelf life (due to lowering the initial load). However, these findings do not allow predictions to be made at other temperatures or under any other conditions. If the temperature changed from 2°C to 3°C the comparison of shelf life becomes invalid and must be repeated for the new temperature regime. The design and use of microbial challenge tests is thoroughly discussed in Notermans *et al* (1993). Predictive microbiology allows the exact effect on the microbial population to be determined providing the main organisms of concern are known. This is particularly important for processors trying to make products more 'natural' by using minimal processing and reducing or eliminating preservatives, in that the reformulations can be tested mathematically, without compromising the safety of the product. At the same time consumers are

purchasing food less frequently and storing it longer (Gibbs & Williams, 1990; Roberts, 1989; Schofield, 1992). Consumers therefore require accurate information on the storage conditions required of the product for it to remain safe. Gibbs & Williams (1990) and Schofield (1992) suggested that one of the more urgent requirements is for consumers to be educated on the correct storage of chilled foods, as food products are often subjected to widely fluctuating conditions in the consumers home.

The term "predictive microbiology" was first proposed by Roberts' group in the UK (Roberts & Jarvis, 1983; Roberts, 1989) in which the growth response of the organisms of concern would be modelled mathematically in respect to the main controlling factors; generally considered to be temperature, water activity and pH (Roberts, 1990), although for some foods other factors may also be important. The idea of being able to predict quantitatively the growth responses of microorganisms has been around for a long time (for example, Jennison, 1935; Scott, 1937), but it was not until the development of computers and the appropriate software with which to analyse the data that predictive microbiology became a recognised field of research.

Jennison (1935) tried to derive quantitative relationships from growth curves by using techniques almost identical to those used today. In his paper of 1935 he disagreed with many of his peers that the bacterial growth curve can be described by using autocatalytic (logistic) functions, which assume a symmetrical growth curve, as his data was aymmetrical about its point of inflection (as is the Gompertz function). Unfortunately he did not develop these ideas further and the argument over which function best describes bacterial growth continues to this day. Scott (1937) had thought through the possible uses of being able to determine the growth rates of microorganisms and stated that

> *"a knowledge of the rates of growth of certain micro-organisms at different temperatures is essential to studies of the spoilage of chilled beef. Having these data it should be possible to predict the relative influence on spoilage exerted by the various organisms at each storage temperature. Further, it would be feasible to predict the possible extent of the changes in populations which various organisms may undergo....".*

The Hazard Analysis and Critical Control Point (HACCP) system was initiated in the 1960's by NASA (National Aeronautics and Space Administration) and the Pillsbury Company who were developing food products for the space program.

One of their objectives was that food products to be used by the astronauts have, as close as possible, to a 100% assurance that they were microbiologically and chemically safe. Traditional methods were found to be inadequate due to lack of control being exercised throughout processing (Sofos, 1993). Processors must assume that pathogens and spoilage organsisms will be present and take appropriate measures to limit the possible extent of growth. This may take the form of refrigeration, drying, salting, the addition of preservatives and fermentation.

The basic steps in the HACCP process as described by the US National Research Council (1985) are

> *"(1) identification and assessment of the hazards associated with growing, harvesting, processing, marketing, preparation and use of a given raw material or food product ; (2) determination of critical control points to control any identifiable hazard; and (3) establishment of systems to monitor critical control points"*

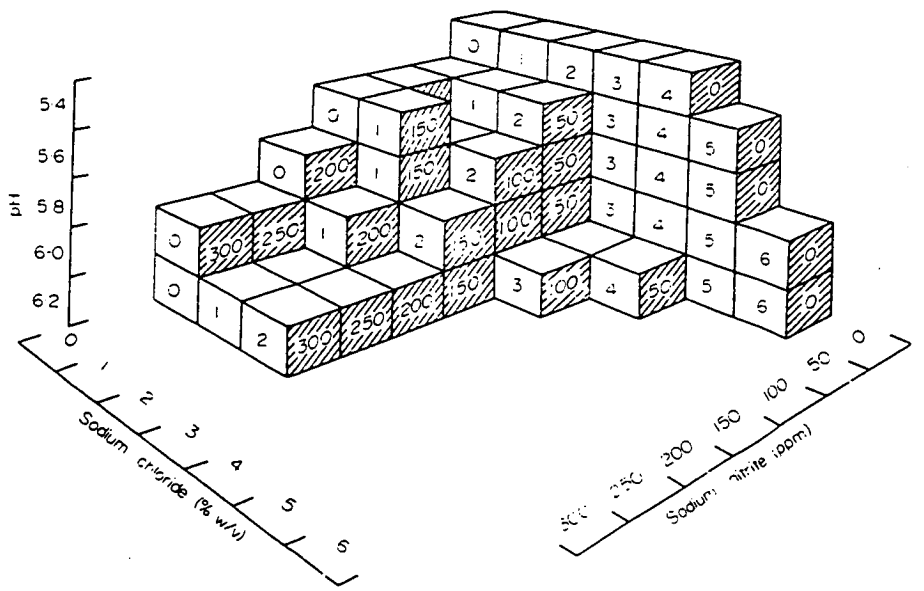
Many regulatory bodies make HACCP a compulsory element in quality systems; for example, Australian Quarantine Inspection Service (AQIS) includes HACCP in the Food Processing Accreditation, Approved Quality Arrangements and Meat Safety Quality Assurances (Sumner, 1995). Despite these regulations Sumner (1995) maintains that any HACCP system is only as good as the level of skill and training of the floor staff. It must therefore be developed from the ground up, involving both operators and supervisors, and must be included in the companies training programs. Further information on how to go about designing and implementing a quality system for the food industry is discussed in Sumner (1995).

HACCP and predictive microbiology are very similar, in that, both are proactive and preventative systems of quality control. When HACCP is properly applied it can be used to control any point in the food system that may contribute to a hazardous situation (Pierson & Corlett, 1992). For example, Gill *et al* (1991a) maintain that many current regulations in the meat industry fail to address the real problems, in that they stipulate a temperature that carcasses must obtain before transportation or demand rates of cooling that are often unachievable without having any justification on which to base the regulations (Ross & McMeekin, 1995). The use of predictive microbiology together with HACCP techniques, could indicate whether the regulations are justified. Incorporation of predictive microbiology techniques into HACCP can reduce the amount of routine microbiology required and could be used to alert personnel that microbiological conditions are not within the 'normal' range.

1.3. MODEL DEVELOPMENT

Predictive microbiology involves the development and application of mathematical models to predict how changes in intrinsic (those attributes that are inherent to the food product, for example, water activity, pH, nutrients and antimicrobial compounds) and extrinsic (qualities that are environmental and include factors such as temperature and water vapour pressure) conditions will affect microbial growth (Mossel, 1983; Gould, 1992; McMeekin & Ross, 1993). The combined effect of these factors determines the growth and composition of microbial biota that will exist and/or grow in a product. Many workers (e.g., Roberts & Ingram, 1973; Roberts, 1990; Ellis *et al*, 1993) have illustrated interactive effects of parameters in the form of 3-dimensional ecograms (Figure 1.2). However these pictorial methods are limited, in that a maximum of three parameters can be included, while mathematical models can include the effects of many combinations simultaneously.

Figure 1.2 An example of a 3-dimensional ecogram showing the effect of pH, NaCl, $NaNO_2$ on the growth of *Clostridium botulinum* type A (NTCC 3806) at 35°C.



(*from* Roberts & Ingram, 1973)

Whiting & Buchanan (1993) proposed a three-tier system of classification of models, in which models are described as being primary, secondary or tertiary. Primary models are those which measure the response of the microorganism to a single set of conditions over time and include growth and inactivation/survival models. Secondary models are described as the response of one or more parameters of a primary model to changes in one or more of the environmental factors, while tertiary models involve the application of secondary models to generate systems for providing predictions e.g., user-friendly software and expert systems.

1.3.1. Primary Models

Primary models are those that describe the bacterial growth curve. The traditional method of determining generation time from the bacterial growth curve, where one log-ten cycle is equal to 3.32 doublings, is shown in Figure 1.3. A major drawback of this method is that it is subjective. By using nonlinear regression techniques to mathematically quantify the parameters of the curve all researchers obtain the same generation times given the same set of data i.e., the process becomes objective.

Roberts (1989) recounted that he selected the modified-Gompertz function because it did not assume a constant growth rate but rather, increases to a maximum and then decreases. The modified-Gompertz function was defined by Gibson *et al* (1987) as

$$L(t) = A + C \exp\{-\exp[-B(t-M)]\} \qquad (1.1)$$

where

L(t)	=	log count of bacteria at time, t	(hr)
A	=	initial level of bacteria	[log(cfu/mL)]
B	=	relative growth rate at M	[(log(cfu/mL))/hr]
C	=	number of log cycles of growth	[log(cfu/mL)]
M	=	time at which the absolute growth rate is maximal	(hr)

From these parameters, the various phases of growth such as length of lag phase, generation time, exponential growth rate and maximum population density can be calculated as

$$\text{Lag Phase Duration} = M - \frac{1}{B} + \frac{\text{Log } N(0) - A}{BC/e} \quad \text{(hr)} \qquad (1.1a)$$

(a corrected form proposed by McMeekin *et al* (1993) to eliminate the problem of the prediction of negative values of lag times as observed by Baranyi *et al* (1993a). When A = Log N(0), then equation (1.1a) reduces to M-[1/B] which is the original form of the equation for lag phase duration put forward by Gibson *et al* (1987)).

Generation Time	= $e$log 2/BC	(hr)	(1.1b)
Exponential Growth Rate	= BC/e	[log(cfu/mL)/hr]	(1.1c)
Maximum Population Density	= A + C	[log(cfu/mL)]	(1.1d)

where

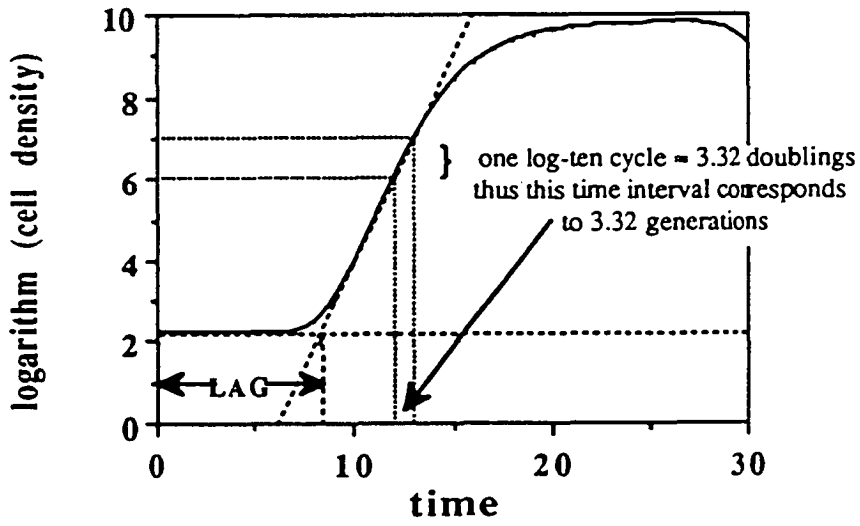
N(0) = cell density at t=0

A, B, C & M as above

Bratchell *et al* (1989) were amongst the first researchers to question the amount of data necessary to fit accurately the modified-Gompertz function. By systematically deleting data from a data set and refitting the modified-Gompertz function, it was shown that the number of curves unable to be fitted increased as the number of points decreased. Gibson *et al* (1988), Labuza & Fu (1993) and McMeekin & Ross (1993) recommended that 10-15 points be the minimum number of readings per growth curve for each independent variable.

A model of similar form is the Logistic function. Its major difference to the modified-Gompertz is that it is symmetrical about M (Gibson *et al*, 1987) while the modified-Gompertz function is asymmetrical. In comparing the modified-Gompertz with the Logistic, various authors (Zwietering *et al*, 1990; Garthright, 1991; Zwietering *et al*, 1992; Willocx *et al*, 1993; Baranyi *et al*, 1993a) have preferred the modified-Gompertz due to its better statistical accuracy and more accurate representation of bacterial growth curves. Many of these same authors have also provided recommendations for various changes in order to improve the modified-Gompertz function. Baranyi (1993a,b) recommended a shift away from empirical models, such as the Gompertz and Logistic functions, and proposed a new model for calculating the specific growth rate in which a non-autonomous differential equation was applied. One potential benefit of the non-autonomous differential equation is that growth curves lacking stationary phase data can still be fitted and an exponential growth rate value obtained.

Figure 1.3 A graphical method for the estimation of generation and lag time from a bacterial population growth curve. The slope of the tangent to the steepest part of the curve estimates exponential growth rate. The generation time can be calculated from this tangent as the time for a 0.301 unit increase in log(cell density), i.e., doubling of the population. The intercept of this tangent with the initial inoculum level is taken as the end of the lag phase.



(Ross, 1993)

1.3.2. Secondary Models

Temperature is one of the main environmental factors affecting shelf life of food products. It is important not only because of its direct effect on the growth rate of microorganisms but because it also impacts on many of the physicochemical characteristics of the environment such as volume, pressure, rates of diffusion and brownian motion (Stotzky, 1974). As a result decreasing temperature is often one of the key methods for increasing the shelf life of food products. Initially researchers tried to model the growth of bacteria over various temperature regimes using the same equations used to model chemical reactions e.g., the van't Hoff rule, which proposes that a 10°C rise in temperature increases the rate of reaction

two to three times. Jennison (1935) found that this was not suitable, as the Q_{10} value changed depending on which 10°C period of the temperature range was chosen, with those at the lower end having much higher Q_{10} values than those nearer the optimum temperature.

Despite the inapplicability of this approach, it was not until the 1970's that various alternatives were suggested. Since then, several mathematical models to describe the effect of temperature on growth rates have been developed and include those broadly classified as Bělehrádek-type models, Arrhenius-type models and Polynominal models. Probability models will also be discussed.

1.3.2.1. Bělehrádek-type Models

Ratkowsky *et al* (1982) proposed a linear relationship based on Ohta & Hirahara (1977) who found a plot of the square root of rate of nucleotide degradation in carp muscle to be linearly related to temperature. The Ratkowsky *et al* (1982) model is suitable for predicting the growth rates of bacterial cultures between the minimum and optimum temperatures and is written in the form

$$\sqrt{r} = b\,(T - T_{min}) \qquad (1.2)$$

where

r = rate at T

T = temperature

T_{min} = notional minimum temperature for growth (i.e., where $\sqrt{r} = 0$)

b = slope of the regression line

Ross (1987) showed that the model proposed by Ratkowsky *et al* (1982) is a special case of the Bělehrádek temperature function, in which the exponent equals two. Ratkowsky *et al* (1983) extended the model to include the entire biokinetic range so that

$$\sqrt{r} = b(T - T_{min})\,\{1 - \exp\,[c(T - T_{max})]\} \qquad (1.3)$$

where

T_{max} = notional maximum for growth analogous to T_{min}

c = coefficient to be estimated

r, T, T_{min}, and b are the same as in equation (1.2)

Lowering water activity is a common method of preserving food. Bacteria attempt to overcome the plasmolytic effect of reduced water activity by accumulating compatible solutes such as proline or glutamic acid intracellularly. McMeekin *et al* (1987) found temperature and a_w to act independently and therefore equation (1.2) could be extended to include a component for water activity by changing 'b' so that

$$b^2 = c\,(a_w - a_{w\,min}) \qquad (1.4)$$

where

c = regression coefficient

a_w = water activity

$a_{w\,min}$ = notional minimum a_w for growth

Substituting b into equation (1.2), the new equation for water activity becomes

$$\sqrt{r} = \sqrt{c}\,(T - T_{min})\sqrt{(a_w - a_{w\,min})} \qquad (1.5)$$

The $a_{w\,min}$ of bacteria varies depending on the humectant used, although generally, growth patterns with solutes such as NaCl, KCl, glucose and sucrose are very similar, while glycerol permits growth at lower a_w values (Prior, 1978). For example, *Pseudomonas fluorescens* has an $a_{w\,min}$ value of 0.957 for NaCl while with glycerol the $a_{w\,min}$ value drops to 0.940 (Prior, 1978). Chandler & McMeekin (1989) found the $a_{w\,min}$, for *Staphylococcus xylosus*, varied from 0.838 for NaCl adjusted media to 0.908 in glycerol . This may be due to the lower amount of osmotic stress caused by glycerol, compared to other solutes, to the bacterial cell and is termed the "solute effect" (Troller, 1987). Despite $a_{w\,min}$ varying with the humectant, the T_{min} remains constant (Chandler & McMeekin, 1989; McMeekin *et al*, 1992) and is an important element in developing parsimonious models.

Various reports in the literature disagree over whether pH can be modelled using the same techniques as for temperature and a_w. Adams *et al* (1991) found the effect of pH on *Listeria monocytogenes* could be modelled although the notional minimum pH for growth (pH_{min}) was acid specific and is due to the differernt pK_a (pK_a = pH at which 50% of the total acid is undissociated) values of the different acids (Raa & Gildberg, 1982). Similar results were shown by Petran & Zottola (1989), Cole *et al* (1990), Wijtzes *et al* (1993), Davey (1994) and Rosso *et al* (1995). However other researchers (Ross, 1993; Passos *et al*, 1993a,b; Buchanan & Klawitter, 1992; Zaika *et al*, 1989; Gibson *et al*, 1988; Vanderzant & Nickelson,

1972) have shown that the generation time is unaffected by pH for at least two pH units and that growth is slower on either side of this plateau.

1.3.2.2. Arrhenius-type Models

Although the Arrhenius equation has been used by many researchers (e.g., Ingraham, 1958; Baig & Hopton, 1969; Mohr & Krawiec, 1980; Reichardt & Morita, 1982), other workers (Scott, 1937; Daud *et al*, 1978; Ratkowsky *et al*, 1982; Phillips & Griffiths, 1987) have found the Arrhenius equation in its simplest form to be inadequate for modelling the growth characteristics of a microorganism. As a result a number of alternative models, based on the Arrhenius equation were developed (Johnson & Lewin, 1946; Hultin, 1955; Sharpe & DeMichele, 1977; Zwietering *et al*, 1991). Schoolfield *et al* (1981) reparameterised the model of Sharpe & DeMichele (1977) in an attempt to overcome the difficulties of fitting the model to data by nonlinear regression. It can be simplified to include only the sub- or super-optimal temperature range, although written in full it is expressed as

$$r(T) = \frac{\rho(25°C)\,\frac{T}{298}\exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{298}-\frac{1}{T}\right)\right]}{1+\exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2L}}-\frac{1}{T}\right)\right]+\exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2H}}-\frac{1}{T}\right)\right]} \qquad (1.6)$$

where

$r(T)$	=	mean rate of development at T; ($time^{-1}$)
T	=	temperature in Kelvin
R	=	universal gas constant
$\rho(25°C)$	=	development rate at 25°C assuming no enzyme inactivation; ($time^{-1}$)
$\Delta H_A^{\neq}$	=	enthalpy of activation of the growth rate controlling reaction
$T_{1/2L}$	=	T at which the enzyme is $^1/_2$ active and $^1/_2$ has been inactivated by low temperature
$T_{1/2H}$	=	T at which the enzyme is $^1/_2$ active and $^1/_2$ has been inactivated by high temperature
ΔH_L	=	change in enthalpy associated with low temperature inactivation of the rate controlling enzyme (cal mol^{-1})
ΔH_H	=	change in enthalpy associated with high temperature inactivation of the rate controlling enzyme (cal mol^{-1})

This model has received the most attention of the Arrhenius-type models, probably due, in part, to its use by Unilever Research in the United Kingdom (Ross, 1993). The Schoolfield model was extended by Broughall *et al* (1983) to include water activity, and later Broughall & Brown (1984) added a further component for pH. Adair *et al* (1989) reparameterised it yet again by expressing the equation with ln(time) rather than rate as the variable in an attempt to improve numerical stability and increase the speed of convergence.

Davey (1989a) proposed another modified and additive Arrhenius-type equation to predict the combined effects of temperature and water activity so that

$$\ln k = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 a_w + C_4 a_w^2 \qquad (1.7)$$

where

a_w = water activity
T = temperature
k = rate
C_0 - C_4 are coefficients to be determined

If the water activity is constant the two last terms in the equation can be omitted. The absence of a cross-product term, such as a_w/T suggests that the effects of a_w and T on ln k are independent of one another (McMeekin *et al*, 1993). McMeekin *et al* (1993) found the ratio of C_3:C_4 to be constant (-1.977 ± 0.02) regardless of the microorganism studied, resulting in their suggestion that the Davey model (equation 1.7) is overparameterised.

1.3.2.3. Polynomial Models

Polynomial equations are a method of fitting an equation to virtually any dataset using as many terms as required. Gibson *et al* (1988) described a polynomial in the form

$$y = a + b_1 s + b_2 t + b_3 p + b_4 s^2 + b_5 t^2 + b_6 p^2 + b_7 st + b_8 sp + b_9 tp + e \qquad (1.8)$$

where

y = response variable (i.e., parameter to be modelled)
e = random error
a, b_1-b_9 are coefficients to be fitted
s, t, p represent NaCl (%w/v), Temp(°C) and pH respectively

to describe the Gompertz parameters A, B, C and M. By taking the natural logarithm of these parameters prior to modelling, negative predicted values were avoided. Gibson & Roberts (1989) found that polynomial equations were not needed for parameters A and C as they did not vary systematically and mean values were substituted into the Gompertz equation. Variations on this method have been used by Bratchell *et al* (1989), Buchanan & Phillips (1990) and Sutherland *et al* (1995). Other researchers related the polynomials directly to the response under study (Cole & Keenan, 1987; Thayer *et al*, 1987; Baker *et al*, 1990; Cole *et al*, 1990). The polynomial approach was used by R.L.Buchanan and colleagues at the USDA/ARS Eastern Regional Research Center, Philadelphia in the development of the Pathogen Modelling Program (PMP) (McMeekin *et al*, 1993).

1.3.2.4. Probability Models

Probability models provide a quantitative assessment of the chance that a particular microbial event will occur within a specified time. This is of most use for pathogens where the rate of growth is less important than the fact that the microorganism is present. Probablity models were originally designed for predicting toxin formation or how many spores of *Clostridium* have survived a particular process ie in the retorting of canned products. The probability of growth detection of *C. botulinum* within a given time was defined as

$$\text{Log}_{10} P(\%) = 5\left(\frac{e^{y}}{1+e^{y}}\right) - 3 \qquad (1.9)$$

where

P	=	probability
y	=	the effect of environmental variables
	=	$b_1 + b_2T + b_3(S_t - LP) + b_4T(S_t - LP)$
T	=	Temperature
S_t	=	elapsed time
LP	=	time to toxigenesis
	=	$a + b_5T + b_6(1/T) + b_7(I)$
$b_1 - b_7$	=	coefficients to be determined
I	=	inoculum concentration

Lindroth & Genigeorgis (1986)

The probability of detectable growth when plotted against time is a sigmoid curve where the upper asymptote represents the maximum probability of growth under infinite time. Ross & McMeekin (1994) consider the sigmoid shape of the probability response to reflect the range of growth / lag-resolution rates, with the fastest rates resulting in the earliest detection of growth.

1.3.2.5. Summary of Secondary Models

Ratkowsky (1993) listed five points which should govern the selection of a model for nonlinear regression, these being

a) parsimony - models should contain as few parameters as possible i.e., the simpler the better
b) parameterisation - which one has the best estimation properties
c) range of applicability - data should cover the entire range under consideration
d) stochastic specification - error term needs to be modelled
e) interpretability - parameters should be meaningful

Given the increasing speed and decreasing cost of technology, parsimony need only be considered when all other aspects of the models in question are equal (Ratkowsky, 1993). The benefits and problems of the various models will not be discussed in this text as numerous articles comparing models can be found in the literature (Stannard *et al*, 1985; Adair *et al*, 1989; Davey, 1989b; Kilsby, 1989; McMeekin *et al*, 1989; Zwietering *et al*, 1991; Alber & Schaffner, 1992, Ross & McMeekin, 1994) while a more historical approach to the development and comparison of kinetic models is covered in Heitzer *et al* 1991, Ratkowsky *et al* 1991 and McMeekin *et al* (1993).

1.3.2.6. Model Validation

The generation times predicted by a model developed in broth media must be compared to the generation times observed in food products and can be termed "validation". This validation process occurs in two stages. Initially the model should be validated in the laboratory. However, it must also be validated in 'real life' situations. That is, it must be tested in industry under normal use, to show that it will accurately predict, in product, under fluctuating environmental conditions. The goodness-of-fit of a model was assessed by Adair *et al* (1989) using the mean square error (MSE), where

$$MSE = \Sigma \frac{\left\{ (obs - pred)^2 \right\}}{n} \qquad (1.10)$$

where

obs = observed value of generation time (hrs)
pred = predicted value of generation time (hrs)
n = number of observations in the dataset

Using MSE, Adair *et al* (1989) found the square root model to show a systematic deviation from the observed values at the lower temperatures. Ratkowsky *et al* (1991) attributed these inconsistencies to the lack of an appropriate transformation of the data ie, when comparing the square root model with observed data the root mean square error (RMSE) should be used to normalise the variance. Little *et al* (1992) disputed the views of Ratkowsky *et al* (1991) maintaining that the number of parameters is largely irrelevant in empirical modelling due to the availablility of modern computing facilities and that the usefulness of MSE depends on the type of comparison required.

Ross (1995) stated that methods for comparing the goodness-of-fit of competing models should " *determine whether a fitted model is statistically acceptable relative to the measuring error inherent in the data*". To compare observed and predicted generation times Ross (1995) introduced to predictive microbiology the use of bias and accuracy factors. The bias factor provides an indication of the average deviation and is described as

$$\text{BIAS factor} = 10^{\frac{\Sigma \log\left(GT_{observed}/GT_{predicted}\right)}{n}} \qquad (1.11)$$

"A value less than one indicates that the predicted generation time is, on average, greater than the observed generation time and is thus 'fail dangerous'. Conversely, a value greater than one indicates that the model is 'fail safe'" (Ross, 1993). Because over- and under- predictions may cancel out, the bias factor provides no indication of the accuracy of the data. Hence, the need for an

$$\text{ACCURACY factor} = 10^{\frac{\Sigma\left|\log\left(GT_{observed}/GT_{predicted}\right)\right|}{n}} \qquad (1.12)$$

"The larger the value, the less accurate is the average estimate. Thus, an accuracy factor of two indicates that the prediction is, on average, a factor of two different from the observed value i.e. either half as large or twice as large, while a value of one indicates that there is perfect agreement between all predicted and observed

values" (Ross, 1993). Ross (1995) cautions that unequal bias, ie systematic overprediction in one region may be balanced by systematic underprediction in another region, may be masked and that another method of assessment, such as a residual plot may be required to verify the bias factor.

1.3.3. Tertiary Modelling

Although models cannot be used outside the range of parameters defined by the model and initially require a large volume of data to derive (Hedges, 1991), they can nonetheless be used in similar situations where the same controlling factors exist, but which were not specifically tested for in the model development (Cole, 1991; Baird-Parker & Kilsby, 1987). A fully validated model offers quantitative information, increased accuracy, objectivity, rapidity, versatility and flexibility to the user (McMeekin & Olley, 1995).

Once a model has been developed, it can be incorporated into a temperature function integrator and the time/temperature history used in conjunction with the model to determine the extent and rate of growth of the organism in question. One of the first time temperature integrators was the *Tefimupot*, (discussed by Olley & Ratkowsky, 1973). Since then a wide range of devices have become available and range from chemical indicators to electronic integrators and temperature loggers. Electronic integrators run as electronic clocks and provide a display of the elapsed shelf life based on relative rates at the recommended temperatures, while loggers record temperature against real time which may then be downloaded as a hard copy and the temperature history interpreted with an appropriate model e.g., Delphi Temperature Logger (Table 1.2). Cost will probably limit such devices to consignments, processes and systems, rather than on individual packages (Ross *et al,* 1993a).

The temperature function integrator should be placed in the area that remains at the highest temperature for the longest period of time provided that no other factor needs to be considered (Gill *et al*, 1991a). Thus probes are placed on the surface of meat carcasses (Gill *et al*, 1991b; Lowry *et al*, 1989; Reichel *et al*, 1991) rather than in the centre where the temperature remains hotter for longer but is generally sterile (Gill, 1979). When using a time/temperature integrator the temperature is recorded at pre-set intervals and the extent of growth that occurred during that interval is determined. Adding the amount of growth during each interval provides the total extent of growth. In graphical terms this can be shown by summing the portion under the graph (Figure 1.4).

Table 1.2 Summary of temperature loggers / integrators commercially available.

Device	Temperature history interpretation as bacterial growth?	Sensor Temperature Range ψ (Min/Max °C)	Number of Channels	Total Record Capacity	Direct Readouts on Device	Operational Life Without Service or Battery Replacement	Approx. Cost ($US)	Manufacturer
Don Whitley TTFI	yes	as needed	1	---	Shelf Life Used	years	700	DON WHITLEY SCIENTIFIC LTD., West Yorks., UK.
Delphi	yes	-20/+40	1	8,000	none	5 years	1000	MANAGEMENT INFORMATION RESOURCES LTD., Wellington NZ.
Smartlog	yes	-10/+39 or as needed	1	32,000	THI*	100 days	900	REMONSYS, Keynsham, Bristol, UK.
Autolog	no	various (-29/+150)	4	5,124	device status	1 years	1000	" " " "
Control One	no	various (-40/+105)	2	250,000	none	10 years	1000	CONTROL ONE, Old Greenwich, Connecticut, USA.
Thinline Datacorder	no	-30/+30	>10?	---	none	60 days	---	CARRIER TRANSICOLD, Syracuse, NY, USA.
Squirrel	no	various (-50/+150)	16	32,000	temp.	6 months	---	GRANT INSTRUMENTS, Barrington, Cambridge, UK.
Thermoking	no	---		---	device status	80 days	---	THERMOKING CORP., Minneapolis, USA.
Anritsu	no	various (-100/+1200)	1-6	3,000	temp.	days	2000	ANRITSU Meter Co., Ltd., Meguro-ku, Tokyo, JAPAN.
DTR(disposable)	no	-25/+35	1	†	temp history	1-8 weeks	25	DTR Co., Ltd., Modesto, California, USA.
Datataker	no	various (-250/+1800)	10-50	300,000	none	18 months	---	DATA ELECTRONICS, Rowville, Vic., AUS.
Auscord	no	various (-50/+1000)	1	†	temp history	20 hours	1000	FOSS ELECTRIC, Terry Hills, NSW, AUS.
ACR	no	various (-40/+125)	1-8	32 000	none	10 years	7-1200	ACR Systems, Inc., British Columbia, CANADA.

ψ The operational range of most devices' recording unit is ≈ -40 to +70°C; external sensors allow wider ranges to be monitored.

† These devices store the information as a continuous paper chart record.

* THI = 'temperature history index' See text for details.

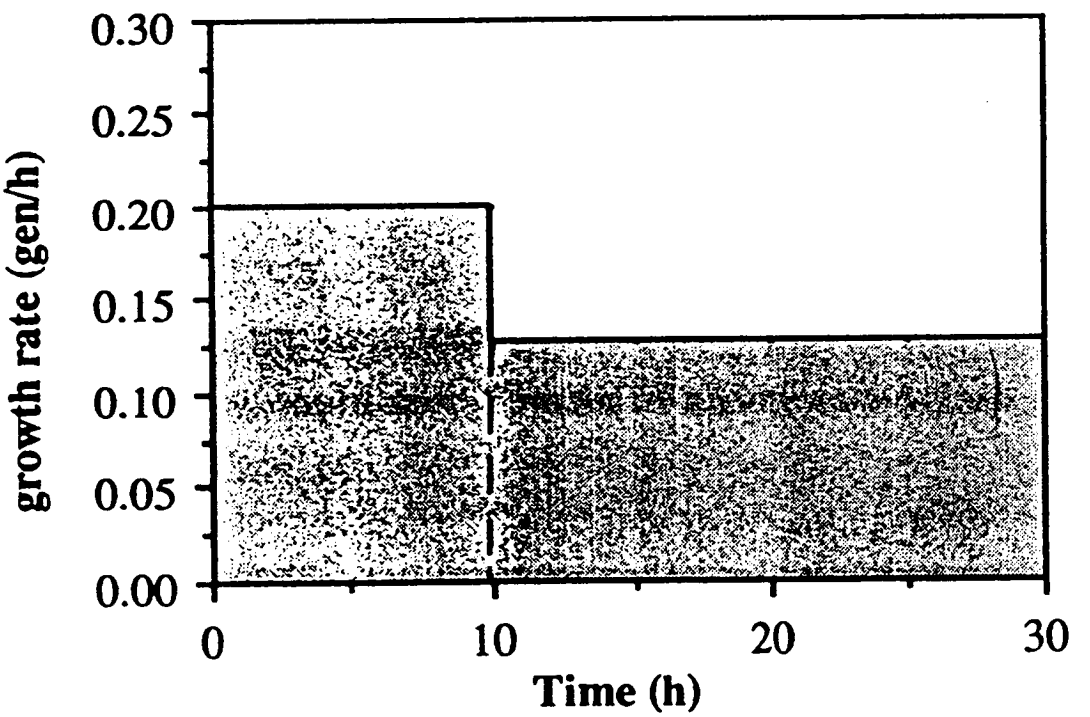
(McMeekin *et al*, 1993)

Figure 1.4

a) Growth rate and its dependence on temperature. The number of generations predicted over the temperature history is given by the product of rate and time. In this example, the temperature fluctuates only once and the calculation is simple, i.e,

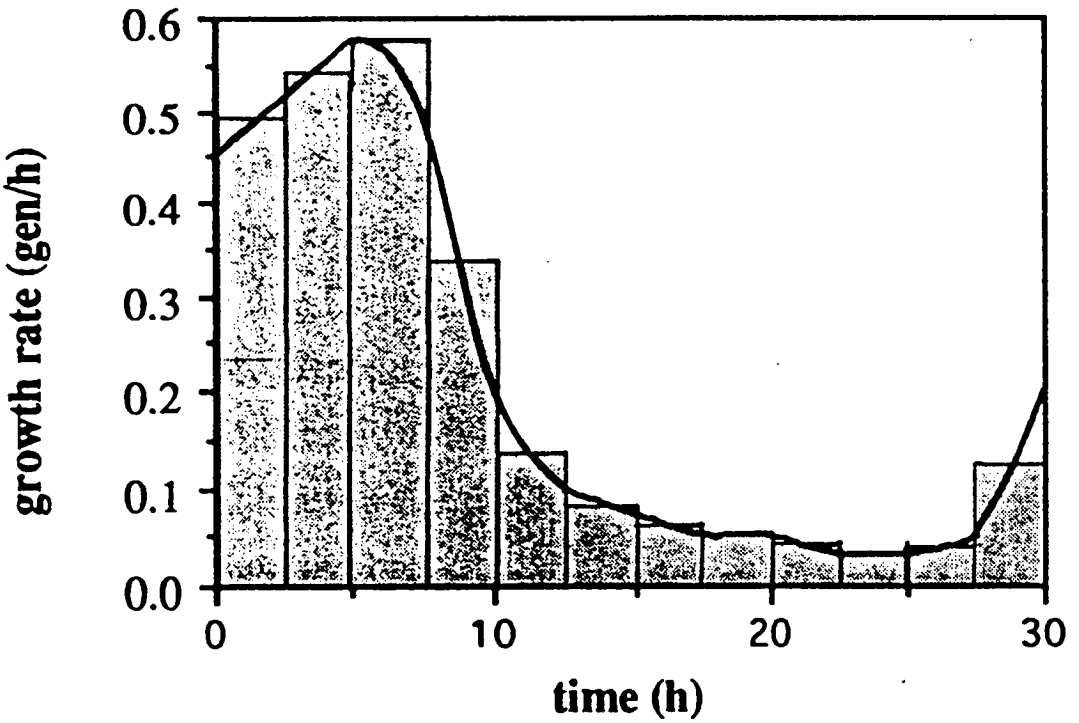
Number of generations = (10 h x 0.20 gen h^{-1}) + (20 h x 0.13 gen h^{-1})
= 2 + 2.6 generations
= 4.6 generations

which is equivalent to an ≈25-fold increase in the population density.



(cont..)

b) In this example, the temperature fluctates rapidly and continuously and the calculation is made more difficult. However, the same approach may still be used to estimate the number of generations by dividing the temperature/rate history into many small time intervals as depicted on the figure and summing the growth predicted in each. As the temperature/growth-rate is continually fluctuating the amount of growth in each interval may be approximated by multiplying the time period by the average of the fastest and slowest in that period. The shorter the time interval chosen the more closely will the estimate approximate the true value.



(Ross, 1993)

Predictive modelling databases currently being developed or in use include the Pathogen Modeling Program with models for *Salmonella*, *Shigella flexneri*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Aeromonas hydrophila*, developed by the Microbial Food Safety Research Unit of the USDA in the USA (Buchanan, 1991, 1993); 'Food Micromodel' produced by the United Kingdom Ministry for Agriculture, Fisheries and Food (MAFF) has models for *A. hydrophila*, *Bacillus cereus*, *Campylobacter* species, *Clostridium botulinum*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* species, *S. aureus* and *Yersinia enterocolitica* (Walker & Jones, 1992, 1993; McClure *et al*, 1994); the FLAIR (Food Linked Agricultural and Industrial Research) program in the EEC with 30 laboratories in 10 countries which aims to set up a European database that will include data and models for both spoilage organisms and pathogens (Cole, 1991; Walker & Jones, 1992); and a decision support system proposed by Zwietering *et al* (1992, 1993) which compares two databases (one for the physical variables of the food products while the other contains the growth data) and matches which organisms are likely to be of concern in a particular product, given the circumstances described by the user. Similar support or expert systems have been discussed by Adair & Briggs (1993), Jones (1993) and Voyer & McKellar (1993).

1.4. CONSIDERATIONS IN MODELLING

1.4.1. Relationship between turbidimetric and viable count methods

The amount of data required to model effectively has resulted in researchers utilising easier, and often indirect, methods of data collection such as turbidimetry in laboratory media under well defined conditions rather than using traditional (viable count) methods. The observation that generation times calculated using turbidimetric methods and viable counts may differ has been recognised for a long time. Monod (1949) commented that although spectrophotometers were becoming widely used since their introduction in 1935, *"not enough efforts have been made to check them against direct estimations of cell concentrations or bacterial densities"*. Monod (1949) also commented that researchers need to determine the effect cell size and media composition have on turbidimetric readings. This concern was later echoed by Maaløe & Kjeldgaard (1966).

1.4.2. Initial inoculum

Mackey & Kerridge (1988) found no effect of inoculum size on the maximum specific growth rates of salmonellae on minced beef. Similar results were shown by Jason (1983) for *Escherichia coli* and Neumeyer (1992) for *Staphylococcus aureus*. Therefore, varying initial numbers will change the absolute time to spoilage but not the relative time. For the purpose of modelling an assumed initial starting inoculum

can be used and is based on either good manufacturing practise (e.g., the dairy industry will invariably produce pasteurised, homogenised milk with an initial level of contamination of <10 pseudomonads per mL (McMeekin & Ross, 1993)) or on the maximum expected value (e.g., worst case scenario (Ross *et al*, 1993a)).

Care should be taken in interpreting the effect of initial microbial load on the length of lag. Observed differences may be due to factors other than the initial load; for example, longer observed lag times may be seen due to the detection time of the equipment, rather than the actual lag time, at lower initial loads.

1.4.3. Modelling Food Systems

Reports by Genigeorgis *et al* (1971) and Raevuori & Genigeorgis (1975) that *Staphylococcus aureus* and *Bacillus cereus*, respectively, grew faster in foods than in media developed a mistrust in some researchers for modelling systems in artificial broth media. These authors continued modelling but used food homogenates for model systems. Other workers find laboratory media based experiments to predict accurately under both constant (Daud *et al*, 1978; Bratchell *et al*, 1989; Ross & McMeekin, 1991; Wijtzes *et al* 1993) and fluctuating (Langeveld & Cuperus, 1980; Lowry *et al*, 1989; Reichel *et al* 1991; Gill *et al*, 1991b; Robins *et al*, 1994) conditions. The differences observed in growth by the earlier researchers may have been due to pH effects rather than to an inherent problem with comparing predictions from broth cultures to those observed in food products. For example, in Raevuori & Genigeorgis (1975) the pH was adjusted using HCl in broths, while in the food products pH was adjusted with glucono-delta-lactone. The different acidulant used may have contributed to the differing growth rates observed.

Mackey & Kerridge (1988) found no interactions affecting the growth of various salmonellae in minced beef although they make the point that it has been reported that *Lactobacillus* may affect growth rates of salmonellae when present under anaerobic conditions. Gill & Newton (1977), Langeveld & Cuperus (1980) and Dainty & Mackey (1992) found the growth rate of one microorganism to be unaffected by the presence of other species except at very high population densities, at which point spoilage or toxigenic dose levels have already been reached (Ross *et al*, 1993a). This implies that each microorganism can be modelled independently without the need to model interactions between organisms. However, interactions may occur in some situations if bacteriocins or other antimicrobial compounds are produced that affect the organisms of concern e.g. nisin, a polypeptide produced by *Streptococcus lactis*, is being used commercially, under the trade name Nisaplin,

for its antibotulinum effect in cheese (Gould, 1992; Davidson & Hoover, 1993); and some lactic acid bacteria such as *Leuconostoc cremoris* and *Streptococcus* may inhibit the growth of *Pseudomonas* (in particular *P. fragi* and *P. putrefaciens* (now called *Shewanella putrefaciens*)) (Babel, 1977; Dubois *et al*, 1979).

1.4.4. Modelling Lag

Lag phase is the period during which microorganisms 'acclimatise' to the new conditions. Food technologists often attempt to extend the shelf life by increasing the lag time (Buchanan & Cygnarowicz, 1990). It is recognised that the temperature history of a culture will have an effect on the length of lag. For example, Walker *et al* (1990) noted that a culture of *Listeria monocytogenes* incubated at 4°C results in a shorter lag than a culture at 30°C when an inoculum from these cultures is transferred to broth at temperatures less than 7°C. This phenomena is described by Ingram & Mackey (1976) as 'cold shock' and is thought to be related to both the rate of cooling and the temperature difference. A consequence of this phenomena is the inability of current models to incorporate accurate components for lag. It may be that because a microorganism is adapted to temperatures experienced in 'real life', smaller estimates of lag time may be needed than for those observed in the laboratory, where cultures are often grown at optimal conditions before transferring the inoculum to the temperature under study (at which point the culture experiences cold shock). However, Chandler & McMeekin (1985a), Griffiths & Phillips (1988b) and Baranyi & Roberts (1994) showed that, provided that all cells had the same pre-inoculation history, the effects of lag could be modelled. Neumeyer (1992) showed preliminary results on *Staphylococcus aureus* that suggested that the effect of lag is dependent on the distance from the optimum temperature. A similar trend is true for the data of Hudson (1993) for *Aeromonas hydrophila*.

It is crucial, particularly for investigations of the lag phase, that experiments be controlled as much as possible during model development. Many researchers have stated that particular effects were observed, when in fact, the pre-inoculation conditions of various cultures differed and the effects described may have been an artefact of the pre-inoculation conditions rather than a true outcome. For example, the square root equation of *Listeria monocytogenes* Scott A, as calculated from generation time data in Ross (1993) is

$$\sqrt{r} = 0.05119 + 0.036637*T \qquad (1.13)$$

where

$\sqrt{r}$ = $\sqrt{}$growth rate (hrs)

T = temperature (°C)

Equation 1.13 was used to calculate the generation times at the temperatures used by Buchanan & Klawitter (1991) and, therefore, the predicted number of generations that elapsed during the inocula incubation. Table 1.3 shows that at 5°C only 7.9 generations had elapsed, while at 37°C 47.5 generations had occurred. Thus although the inocula was in exponential growth phase when transferred from the 5°C inocula, it was well into stationary phase for the 42°C inocula. The extended LPD observed by Buchanan & Klawitter (1991) in the transfer of an inocula from 42°C to 5°C may therefore be due to the condition of the inocula rather than as a direct consequence of the temperature shift. A similar comparison has been reported in McMeekin *et al* (1993), in which the predicted generations varied somewhat from those in Table 1.3. These differences are due to McMeekin *et al* (1993) calculating the generation time of *L. monocytogenes* from data of Buchanan *et al* (1989) rather than Ross (1993).

Table 1.3 The predicted number of generations expected to occur at the temperature and duration of incubation used by Buchanan & Klawitter (1991) for *L.monocytogenes* Scott A data.

Temperature (°C) of incubation	Duration of incubation (hr)	GT (hrs) predicted from Eq.1.13	Number of generations
5	144	18.20	7.9
10	96	5.74	16.7
13	96	3.59	26.7
19	48	1.79	26.8
28	24	0.86	27.8
37	24	0.50	47.5
42	24	0.40	60.7

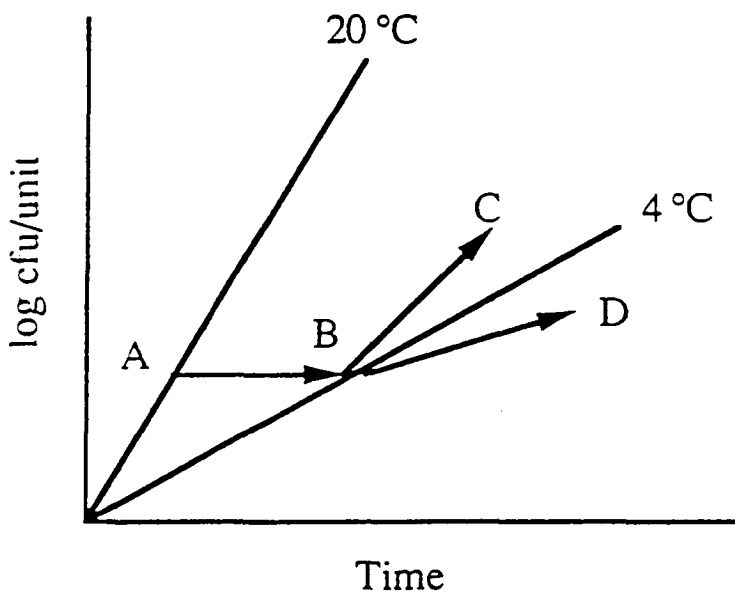
Note:

$$\text{Predicted Number of Generations} = \frac{\text{Duration of Incubation}}{\text{Generation Time}}$$

1.4.5. Temperature History Effects

Some authors have suggested that the previous temperature history may effect the growth kinetics. This is shown diagrammatically in Figure 1.5 where an organism is grown at 20°C to level A and is then transferred to 4°C (level B). If there are no history effects the culture would continue growing at the same rate as a culture initially started at 4°C. However, if a positive history effect occurs it will grow faster (B → C) while a negative history effect results in slower growth (B → D). If history effects occur any modelling will become less reliable as the temperature history is often unknown.

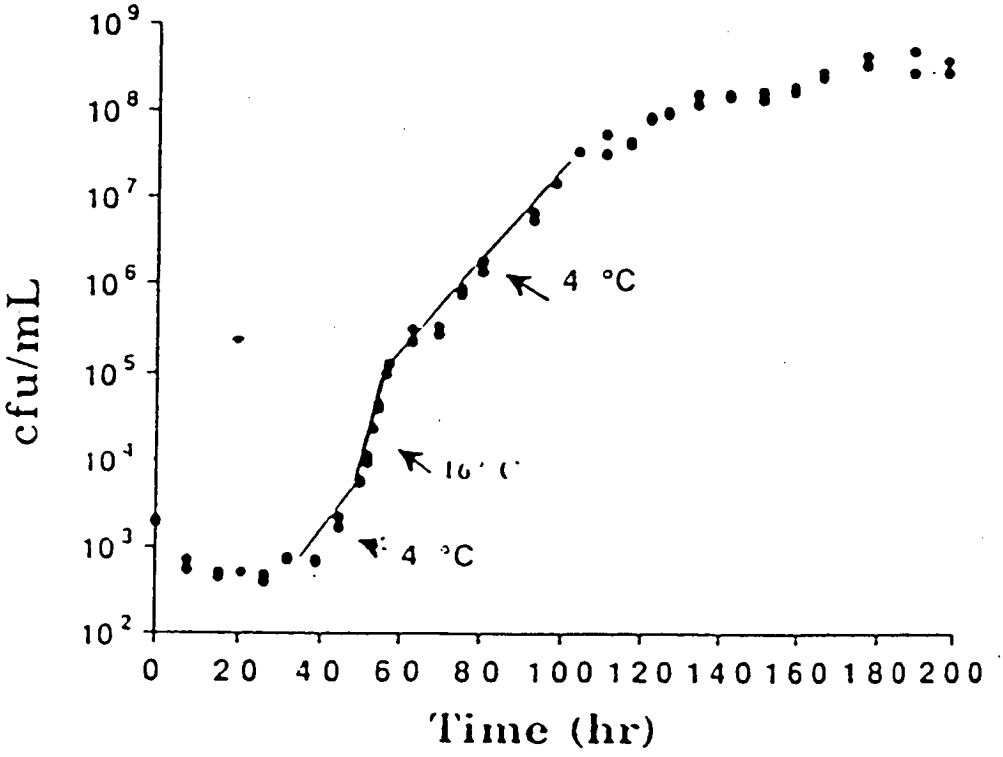
Figure 1.5 Temperature history effects when product is shifted after exposure at one temperature (A) to a second temperature (B). If no effect occurs, the results are similar to always being at the second temperature. The other lines represent a faster rate (positive history effect B → C) and a slower rate (negative history effect B → D).



(Labuza *et al*, 1992)

Although there may be some history effect on the lag phase (Walker *et al* 1990; Buchanan & Klawitter, 1991; Fu *et al*, 1991) most researchers find no such effect on the growth rate (Buchanan & Klawitter, 1991; Neumeyer, 1992; Hudson, 1993). Maaløe and Kjeldgaard (1966) state that "*one should observe a culture through whatever lag it may exhibit, and not be satisfied until at least three doublings at a constant rate have been registered.*" Often this is not observed and may result in authors attributing a temperature history effect on the growth rate of a culture when, in fact, none exists. For example, in Figure 1.6, Fu *et al* (1991) fluctuates a culture of *P. fragi* from 4°C to 16°C and back to 4°C. The culture was barely out of lag phase at 4°C, and therefore had not achieved three doublings at a constant rate, before it was moved to 16°C and then back to 4°C. Given these conditions the proposition by Fu *et al* (1991) that a temperature history effect was observed cannot be considered valid. Jennison (1935) acclimatised his cultures to the temperatures under study (22, 27, 32, 37°C) for four months (with weekly transfers) and still found no difference in growth rates to those kept at 22°C and

Figure 1.6 *P. fragi* growth curve under the abrupt temperature transition of 4 - 16 - 4°C.



(Fu *et al*, 1991)

then tested at the four temperatures. To quote Jennison (1935) the

> *"rate of reproduction at a given temperature was the same whether the cultures used for inoculation were acclimatized to that temperature or to room temperature"*

Lark & Maaløe (1954), Ng *et al* (1962), Shaw (1967), Shaw *et al* (1971), Yamamori *et al* (1978) and Wu & Welker (1991) found that when large abrupt temperature shifts occurred the culture experienced an immediate lag followed by a transitional rate that was intermediate between the rates expected for the initial and final temperatures, before attaining the rate expected at that temperature. This effect was most pronounced in shifts down in temperature (rather than shifts up) and when the temperature difference was greater than about 10°C. This could be explained in two ways a) by the fact that the individual cells will not recover at exactly the same rate i.e., when measuring rate and generation times we are looking at the culture as a whole and not at individual cells; and b) Yamamori *et al* (1978) and Wu & Welker (1991) found the adaptation or transient period to correspond to the de novo synthesis of some proteins.

1.4.6. Fluctuating Temperature Conditions

Models are developed using cultures at constant conditions. In practice, foods are subject to fluctating conditions and models must predict accurately for all situations. While many studies (Blankenship *et al*, 1988; Mitchell *et al*, 1995; Langeveld & Cuperus, 1980; Smith, 1987; Lowry *et al*, 1989; Reichel *et al*, 1991; Gill *et al*, 1991b) show that models predict accurately under fluctating temperature regimes, frequent and abrupt transitions, so that transitional rates represent a large portion of the storage history, may result in unreliable predictions. Brocklehurst *et al* (1995) studied the effect that sinusoidally-varying temperatures, between 4 and 22°C and 12 and 22°C in the range 12 to 480 minutes, had on the growth of *Salmonella typhimurium* LT2. As the cycle period decreased the discrepancy between observed and predicted values increased such that, in the temperature range 4 to 22°C, observed and predicted generation times differed by 35%. Similar trends were observed as the cells became more stressed e.g. at low pH (5.0), higher NaCl (3.5%) or low temperature (4°C rather than 12°C). Studies by Baranyi *et al* (1995) show that the effect of frequent and abrupt transitions may only be of concern in some situations. While temperatures fluctuated between 25 and 5°C observed and predicted values for *Brochothrix thermosphacta* agreed but once the temperature fluctated from 25°C to 2.8°C significant deviations from the model occurred. Baranyi *et al* (1995) attributes the deviation to an additional lag period induced by the cold shock experienced by the culture.

1.5. USE IN INDUSTRY

Jarvis (1983) in a "Delphi forecast", indicated that most microbiologists thought that by the end of the century the traditional methods of enumerating bacteria would be superseded by automated and mechanised procedures. However they did not agree on the acceptability of the alternative methodologies. In the specific case of predictive microbiology, only 25% of panellists considered that it may be used by industry by 1996. The current limited acceptance of predictive microbiology by industry is probably due to the lack of appropriate software.

In addition to increasing the speed of product quality estimates, predictive microbiology reduces the amount of laboratory analysis required, particularly end product testing. In this regard, it can be a useful tool in HACCP procedures by providing an objective assessment of a process or allowing comparisons with other processes. For example, Gill & Jones (1992a) used an integrated approach to assess the hygienic efficiency of two commercial methods for cooling pig carcasses and Gill & Jones (1992b) studied the storage efficiency of vacuum-packaged beef from in-plant storage through to final arrival of the consignments at the shop front. They found the main problem area was in loading warm consignments into the refrigerated trucks for transport resulting in products that only achieved 25% of the potential shelf-life.

Cherng & Zall (1989) make the point that most shops rotate stock on a first-in first-out basis. However, for perishable products such as milk, such a system may result in temperature abused stock being placed at the back of the shelf while better quality milk with an earlier use-by date is placed at the front. If temperature histories were monitored throughout the processing and distribution chain, the temperature abused items could be switched to the front of the shelf and therefore be sold first. Although a temperature recording device alone (such as a time-temperature indicator) will give an indication of temperature abuse, it will not provide information (as predictive microbiology will) on the effect on the length of shelf life of the product.

To take this idea one step further would be the use of predictive microbiology to settle insurance claims by including time/temperature monitors with large consignments throughout the distribution chain. Not only can the extent of damage (and therefore the financial loss) be determined but also at what point the damage occurred; and therefore the person or company who is at fault can be identified.

Due to the current expense of temperature logging units, it is not possible to install time/temperature integrators on all components of a processing chain; for example, tankers and 'on farm' cooling systems in the dairy industry. They can, however, be used as an education tool to show farmers how adherence to simple cooling techniques can increase the quality of milk with very little investment or as a quantitative assessment of the financial viability of new equipment. Given that some dairy factories are now paying farmers for the quality for their milk, farmers are interested in achieving better quality milk to get the highest price possible.

This technology provides a rapid, cost-effective, non-invasive objective quality assessment, which may be particularly useful in areas where microbiological expertise is limited (e.g., in developing areas) (Ross & McMeekin, 1991) and provides power, objectivity and precision in the day-to-day decision making for microbiologists.

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2. AIMS

Psychrotrophic pseudomonads are the major spoilage organisms of food products with neutral pH, high water activity and a short life, such as milk and cream (Chandler & McMeekin, 1985a; Shelley *et al*, 1987; Griffiths *et al*, 1988; Chandler *et al*, 1990; Tatini *et al*, 1991). By developing a model for the growth of psychrotrophic pseudomonads the rate of spoilage of various products can be determined simply by monitoring the product temperature. Benefits of predictive modelling are numerous and include predicting shelf life, assessing the hygienic efficiency of processing and distribution, determining the effect of lapses in storage conditions and determining the microbiological quality and safety of a product.

The aim of the project is to develop a model for predicting the growth of psychrotrophic pseudomonads under fluctuating temperature conditions and over a range of relevant water activities. The resultant model is to be incorporated into a computer software package that industry can use easily.

2.1. Effects of Temperature on Growth

Psychrotrophic pseudomonads isolated from milk and obtained from CSIRO Dairy Research Laboratories in Victoria will be used. It is proposed to develop the psychrotrophic pseudomonad model using a 'worst case' approach, in which the fastest strain of dairy origin is used to develop the model, and thus represents the worst that can happen ie, other strains will grow slower and thus the model will predict in a 'fail safe' fashion The fastest strain will be selected by growing all the pseudomonad strains at ≈10°C this being an abuse temperature often encountered by dairy products.

Growth rates will be determined by growing the strains in artificial broth media and monitoring growth using turbidimetric methods. Growth rates will be calculated from parameters derived from the Gompertz function (equation 1.1). The resultant growth rates will be described as a square root model.

This process will be repeated with a number of other strains including a cocktail of 5 strains in which both the fastest and slowest strains are included. This is necessary in order to ascertain that the fastest strain dominates in a mixed culture and generates a growth rate that is the same as the dominant strain in axenic culture.

2.2. Effects of Water Activity on Growth

Factors other than temperature often affect the growth rate of pseudomonads in many food products. Lowering water activity is commonly used as a means of preservation in order to slow the growth of microorganisms. McMeekin *et al* (1987) found that a variant of the square root model could also be applied to water activity. Water activity models will be developed, using sodium chloride as the humectant, for various pseudomonad strains.

2.3. Model Calibration

In predictive microbiology large numbers of growth rate estimates are required and thus methods that are less labour and time intensive are desirable, hence the use of turbidimetric methods to monitor culture density. An attempt will be made to resolve the discrepancy between generation times calculated from turbidimetric methods may be different to those from viable count (VC) methods by comparing growth of cultures using both turbidimetric and VC methods. VCs are the standard method for enumerating bacteria in food microbiology and therefore any modelling exercise must describe the extent of growth in VC equivalents, rather than that observed by indirect methods.

2.4. Model Validation

The temperature/water activity model for psychrotrophic pseudomonads will be developed in artificial broth media using turbidimetric methods and calibrated in terms of VC. It is, therefore, necessary to compare the growth rates observed in various products with those predicted by the model.

Initial validations will be performed in the laboratory under rigorously controlled conditions, so that apart from the substrate, environmental factors are as well controlled as possible during model development. Where pseudomonads are undetectable in the product, the strain used to generate the model will be inoculated into the product. The observed growth rates will then be compared to those predicted by the model.

After validation under laboratory conditions, trials in industry will be conducted. The model will be incorporated into a computer software spreadsheet and sent to various industry, educational and research institutions for trialing. They will be asked to monitor both the temperature, using an electronic data logger, and the pseudomonad counts for various processes. The data will then be analysed by the candidate. As with the laboratory studies, the observed growth rates will be compared to those predicted by the model.

In order for predictive models to be used by industry, they must be presented in a form that is user-friendly. To determine this industry participants will be sent a questionnaire to determine the usefulness and usability of the software and manual.

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3. METHODS

3.1. MATERIALS

All equipment, media and culture (identification and storage) methods used in this study are described in Appendix 1.

3.2. PRELIMINARY STUDIES

3.2.1. Isolation and Identification of Pseudomonads

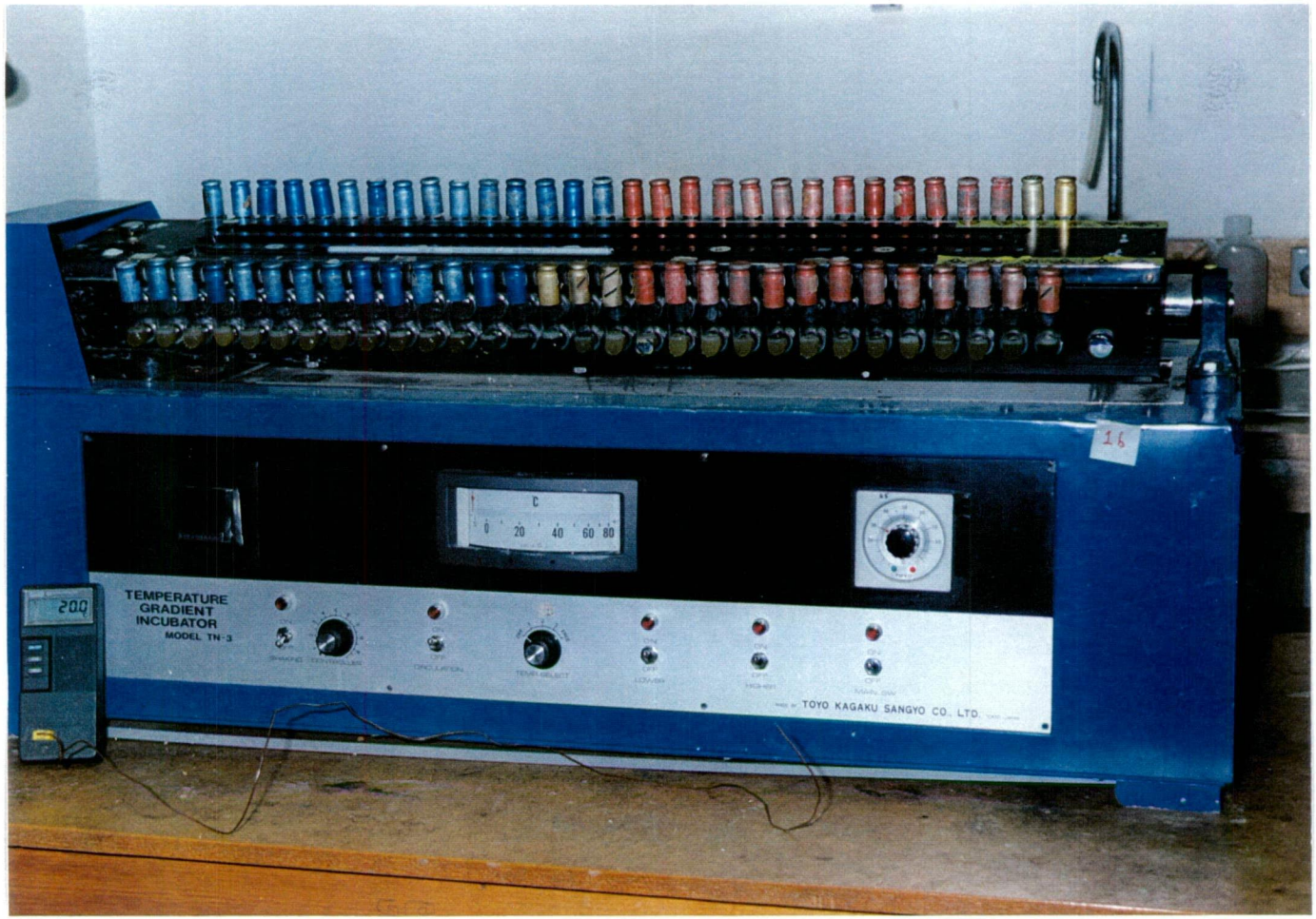
Nine strains of psychrotrophic pseudomonads of dairy origin were obtained from the CSIRO Dairy Research Laboratories in Victoria. Other pseudomonads were isolated from pasteurised modified milk by allowing cartons, purchased at retail outlets, to spoil at 4 and 10°C. 1mL samples were removed at spoilage, diluted using serial decimal reduction in 0.1% peptone, plated onto *Pseudomonas* Selective Agar (PSA) and incubated at 25°C for 2 days.

Representative colonies were plated onto Plate Count Agar (PCA) and confirmed, or otherwise, as pseudomonads using the gram stain, oxidase reaction, oxidation / fermentation and motility tests. Attempts were made to identify pseudomonad isolates to species level using gelatin hydrolysis, King's B Medium for Fluorescein b, 0/129 sensitivity, casein hydrolysis and growth on various carbohydrates (glycerol, xylose, trehalose, m-inositol and levan production). *Staphylococcus aureus* 3b and *Escherichia coli* M23 were used as controls. Information on the identity of isolates was also obtained using Analytical Profile Index (API) 20NE strips. Isolates were maintained in both short term storage for 1-2 months (using slopes of PCA) and long term storage (frozen on beads at -70°C). Full details of all identification and storage methods are described in Appendix 1.

3.2.2. Strain Selection

The fastest growing strains will be the most significant in the spoilage association of milk and dairy products. To obtain an indication of the range of growth rates, and to select the strains for detailed modelling studies, the growth rates of the psychrotrophic pseudomonad strains were determined by growing the isolates in Nutrient Broth No.2 at 10.8°C (this was the lowest constant temperature the TGI could maintain isothermally in a 10°C room) on the Temperature Gradient Incubator (TGI) (Figure 3.1), using the protocols described in 3.3.1. Growth was monitored using turbidimetric methods and generation times calculated using the protocols described in 3.3.1.

Figure 3.1 The Temperature Gradient Incubator



3.3. MODEL DEVELOPMENT

3.3.1. Temperature Models

3.3.1.1. Preparation of Inocula

Cultures to be used as inocula were grown at 25°C for 2 days in 30mL Nutrient Broth No.2 in a 150mL flask thereby ensuring cultures to be in the stationary phase e.g., assuming an initial inoculum of 10^3 cfu/mL, a final maximum population density of 10^{10} cfu/mL and a generation time of 44 minutes at 25°C (refer to Figure 4.2), results in a culture at least the equivalent of 40 generations into stationary phase with the inocula for each experiment having been exposed to the same pre-inoculation conditions.

3.3.1.2. Temperature Gradient Incubator Protocols

L-tubes containing 10mL Nutrient Broth were sterilised and placed on the TGI approximately 12 hours prior to inoculation to allow the TGI to equilibrate and for the media to reach the required temperature. Sufficient inoculum was added to each L-tube to reduce the %Transmittance (%T) to ≈80% (usually 200-300 µL). The amount of inoculum added was constant for each experiment on the TGI. The time of inoculation was considered zero time. The TGI was operated in a constant temperature room to minimise temperature fluctuations within each L-tube which were maintained at ± 0.5°C. L-tubes were agitated (≈40 oscillations per minute) to minimise the formation of oxygen gradients. At the end of the growth curve, the temperature in each tube was recorded 5 times (at approximately one generation time intervals) using an electronic Fluke thermometer.

3.3.1.3. Turbidimetric Measurements

At periodic intervals after inoculation, time (since inoculation) was recorded and, %T readings taken using a spectrophotometer at 540nm. Within the range 20 to 60 %T is linear and a doubling of the population is represented by a 24.5% decrease in %T (Ross, 1993). By plotting change in %T versus time, a sigmoid curve results from which the generation times were determined using the Gompertz function described in 1.5.1. Adjusting Equation 1.1b,

$$\begin{aligned} \text{generation time (\%T)} &= 24.5e / BC \\ &= (24.5 * 2.71828) / BC \\ &= 66.60 / BC \end{aligned} \qquad (3.1)$$

Likewise

generation time (VC) $= e\log 2/BC$

$= (0.30103 * 2.71828) / BC$

$= 0.81828 / BC$ (3.2)

Generation times were described in 'minutes' unless otherwise specified. Growth curves were considered complete when the %T had dropped to 5-7%. A minimum of 15 readings were recorded per growth curve.

3.3.1.4. Model Generation

Although *P. putida* 1442 was the primary strain used for modelling purposes, a number of other strains (*P. fragi* NCIMB 8542, *P. fragi* I6, *P. fluorescens* 1412 and a cocktail of five strains consisting of *P. putida* 1442, *P. fragi* I6, *P. fluorescens* 1412, *P. fluorescens* I1 and *P. fluorescens* I8.2) were also modelled in artificial broth media (Nutrient Broth No.2) at approximately 1°C (for 0 - 30°C temperature range) or 0.5°C (for 0 - 15°C temperature range) intervals to determine the T_{min} and b values of a number of strains. Regression equations were fitted using Cricket Graph and the square root model of Ratkowsky *et al* (1982) (Equation 1.2).

P. putida 1442 was also grown over the temperature range 20 - 50°C to determine the growth rate response over the entire biokinetic range using the model described in Equation 1.3. The non-linear regression curve was fitted using UltraFit.

3.3.2. Water Activity Models

3.3.2.1. Preparation of Inocula

Inocula were prepared as described in 3.3.1.1.

3.3.2.2. Preparation of Media

500mL of Nutrient Broth No.2 (Broth A; 0 %NaCl (w/w)) was prepared. A second batch of Nutrient broth No.2 that included 40g NaCl was prepared, made up to 450mL, the pH checked and then filled to 500mL volumetrically (Broth B; 8.00 %NaCl (w/w)). Both broths were autoclaved at 105°C for 30 minutes. Capped L-tubes, 10mL and 1mL pipette tips were also sterilised at 121°C for 15 minutes.

3.3.2.3. Calculation of Water Activity

Using data from Table 2 in Resnik & Chirife (1988), %NaCl (w/w) was plotted against a_w to obtain an equation relating water activity and %NaCl (Figure 3.2). Broths A and B were mixed aseptically, in sterile L-tubes, in various ratios to achieve the range of water activities required (ie, from 0.9995 to 0.9499). The volume of inoculum added must also be included in calculations for the %NaCl of each L-tube. %NaCl was therefore calculated as

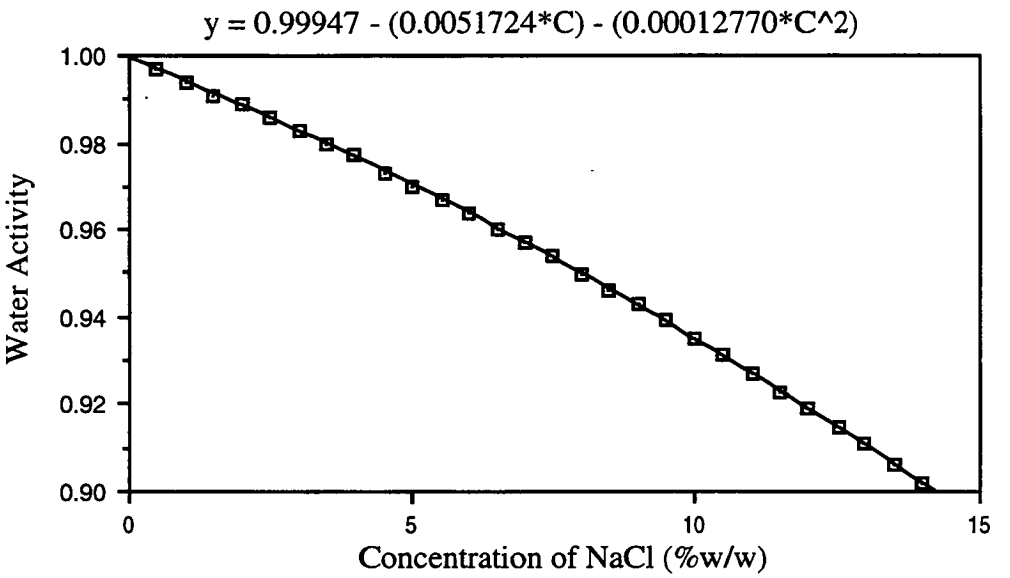
$$\%\text{NaCl (w/w)} = \left(\frac{B}{A+B+C}\right) \text{x Max \%NaCl} \qquad (3.3)$$

where A = mls of Broth A
B = mls of Broth B
C = mls of inoculum (grown in Broth A)
Max %NaCl = 8

The water activity of each L-tube was calculated, from the equation produced in Figure 3.2, using the %NaCl calculated from Equation 3.3. This value was then multiplied by 0.9966 (to take into account the humectant content already present in the Nutrient Broth) to give the true water activity (Robinson & Stokes, 1949).

The mathematically derived water activity was compared to the water activity (a_w) determined using an Aqualab CX2 (Decagon Devices, Pullman, Washington, USA)which uses a cooled mirror dewpoint technique for measuring a_w. The comparison was carried out at five water activities as shown in Table 3.1. Each a_w determination was repeated five times.

Figure 3.2 The relationship between water activity and salt concentration (*data from* Resnik & Chirife, 1988)



y = water activity
C = % NaCl (w/w)

Table 3.1 Proportions of Broths A and B mixed together and the mathematically calculated a_w of that mixture.

mLs of Broth		a_w
A	B	
15	0	0.9961
12	3	0.9875
8	7	0.9751
4	11	0.9614
0	15	0.9467

3.3.2.4. Isothermal Temperature Gradient Incubator Protocols

For modelling purposes the growth rate at each water activity was determined in duplicate and the generation times calculated from duplicate tubes averaged. Although the TGI was set isothermally at 19°C, the temperature varied (±0.5°C) for each isolate depending on the amount of other equipment in use in the room at the time. To compare slopes of the square root plots obtained from each isolate tested, all generation times were calculated as those at 20°C using the relative rate concept where

$$\text{Rate of } T_y \text{ relative to } T_x = \left(\frac{T_x - T_{min}}{T_y - T_{min}} \right)^2 \qquad (3.4)$$

McMeekin *et al* (1988)

where T_x is the temperature of concern,
T_y is 20°C and
T_{min} of *P. putida* 1442

The TGI was set up and growth curves monitored as described in 3.1.3.2. and 3.1.3.3.

3.3.2.5. Model Generation

Generation times (GT) were calculated from parameters derived from the Gompertz function discussed in 1.5.1 and were described in 'minutes'. Regression equations were fitted using Cricket Graph and the model of McMeekin *et al* (1987) in which

$$r = c\,(a_w - a_{w\,min}) \qquad (3.5)$$

where

- r = growth rate
- a_w = water activity
- $a_{w\,min}$ = notional minimum a_w for growth (i.e., where r=0)
- c = slope of the regression line

Water activity models were developed for *P. putida* 1442, *P. fragi* NCIMB 8542, *P. fluorescens* 1412, *P. putida* 1261 and *P. fluorescens* 8.2.

3.3.3. Combined Temperature / Water Activity Models

L-tubes containing 15mL Nutrient Broth No.2 at water activities of 0.996, 0.977, 0.969 and 0.960 (prepared according to the procedure in 3.3.2.) were placed on the TGI over a temperature range of 0 - 30°C. Prepared broths were inoculated with *P. putida* 1442 and growth monitored using turbidimetric methods as described in 3.3.1. The experiment was repeated using *P. fluorescens* 1412. Regression equations were fitted using Cricket Graph and the combined temperature / a_w model described in equation 1.5.

3.3.4. Effect of pH on the Growth of Pseudomonads

Two sets of 6.3g Nutrient Broth No.2 powder and 5.113g Lactate (to make a final solution of 0.2M Lactate) were dissolved in 200mL distilled water and autoclaved in 500mL flasks (at 121°C for 15 minutes) together with capped L-tubes, 200mL distilled water, 10mL, 1mL and 0.1mL pipette tips, NaOH and HCl solutions.

One flask of nutrient broth solution was adjusted to pH 4.5 (Broth A), while the other was adjusted to pH 8.5 (Broth B), using the NaOH and HCl solutions. Sterile distilled water was added to the broths to bring each to 500mL volumetrically. Broths A and B were mixed in varying proportions in sterile L-tubes to achieve a range of pH between 4.9 and 7.8.

The L-tubes were placed in the TGI set at 19.1°C. Tubes were inoculated with *P. putida* 1442 and pH recorded. Experiments were set up, growth curves monitored (using turbidimetric methods) and generation times calculated according to the procedures described in 3.3.1. Tubes were discarded after 7 days if no growth (ie no decrease in %T) was observed.

3.4. MODEL CALIBRATION

3.4.1. Viable Count Measurements

At each sample time, 0.1mL aliquots were removed and diluted in 9.9mL 0.1% peptone followed by serial decimal dilutions prepared in 9mL, 0.1% peptone. 0.1mL of the three highest dilutions were plated, in duplicate, onto PCA. After incubation at 25°C for 2 days plates with between 30 and 300 colony forming units (cfu) were counted. Log cfu/mL values were calculated using the method originally described by Farmiloe *et al* (1954) and used in the MAFF protocols (Anon, 1990) in which

$$\text{CFU / mL or gram} = \frac{C}{V\left(n_1 + 0.1n_2\right)f} \qquad (3.6)$$

where

C = total number of colonies counted
V = volume of inoculum applied to each plate
n_1 = number of plates counted at the lower dilution
n_2 = number of plates counted at the higher dilution
f = dilution factor for the higher dilution

An average of 10 - 15 samples were taken throughout the growth curve. Note that fewer readings were taken compared to the turbidimetric method because of time constraints involved in preparing the media, taking, plating and reading the results of samples.

3.4.2. Correlation of viable count and turbidimetric methods

P. putida 1442 was selected as a representative test organism (refer to 3.2.2.) and the inoculum prepared using techniques described in 3.3.1.1. The inoculum was serially diluted from log 9.5 cfu/mL (the maximum population density) so that L-tubes, containing 15mL Nutrient Broth No. 2, had an initial inoculum level of approximately log 5 cfu/mL. During growth, both %T and VC were monitored as described in 3.3.1. and 3.4.1. respectively. Generation times were calculated as in 3.3.1.4.

3.5. MODEL VALIDATION (LABORATORY STUDIES)

3.5.1. Milk

3.5.1.1. Aerated

Pasteurised modified and homogenised whole milks were obtained from a local supermarket and plated immediately onto PCA and PSA in order to determine the initial load of both total viable and pseudomonad counts. If viable counts were less than log 1.0 cfu/mL, the milk was inoculated with *P. putida* 1442 to have an accurately detectable initial inoculum level of approximately log 5 cfu/mL. Raw milk was obtained from a local milk manufacturer and the growth of the natural microbiota monitored.

10mL of milk was placed into each L-tube and allowed to equilibrate on the TGI at various temperatures. Aliquots were plated onto PCA for the inoculated milks with occasional samples (early, middle and end of the growth curve) plated onto PSA to check the dominance of the *P. putida* 1442 culture. Raw milk was plated

onto both PCA and PSA. pH was monitored throughout the growth curve for all the milks. Experiments were set up, monitored using viable count methods and generation times calculated according to the procedures described in 3.3.1. and 3.4.1.

3.5.1.2. Minimal Agitation

300mL of pasteurised modified milk were placed in a number of sterile 500mL Schott bottles, inoculated with *P. putida* 1442 to initial levels of approximately 10^5 cfu/mL and incubated at various temperatures. At sampling times, the Schott bottles were tipped 180° (simulating pouring milk from a carton) resulting in minimal and intermittent aeration. Experiments were set up, monitored using viable count methods and generation times calculated according to the procedures described in 3.3.1. and 3.4.1.

3.5.2. Evaporated Milk

Pseudomonads are not spoilge organisms of evaporated milk. Evaporated milk, was however, considered useful as a low water activity substrate for model validation. Evaporated milk was obtained from a local supermarket and plated onto both PCA and PSA in order to determine the initial load of both total viable and pseudomonad counts. As both were less than log 1.0 cfu/mL, the evaporated milk was inoculated with *P. putida* 1442, to initial levels of approximately log 5.0 cfu/mL, in order to have an easily detectable inoculum. The a_w was determined using an Aqualab CX2. Trials were then carried out as described for pasteurised milk in 3.5.1.1. on PCA and, at six of the temperatures, on PSA throughout the growth curve.

3.5.3. Cream

Two brands of cream (35% fat) were bought at a local shop, plated onto PCA and PSA as in 3.5.2., and the water activity determined using an Aqualab CX2. Brand 1 had an initial load less than log 1.0 cfu/mL and was inoculated with *P. putida* 1442 to levels of approximately 10^5cfu/mL. Brand 2 contained detectable levels of pseudomonads and so the natural microbiota was monitored. For each brand 40mL samples of cream were put into 250mL Erlenmeyer flasks and placed at various temperatures (2, 6, 11, & 15°C) in Lauda waterbaths. Growth was monitored by removing 1mL aliquots and plating onto PCA (Brand 2 only) and PSA (both brands) using the methods described in 3.5.1.1. Generation times were calculated as described in 3.3.1.4.

3.6. MODEL VALIDATION (INDUSTRY STUDIES)

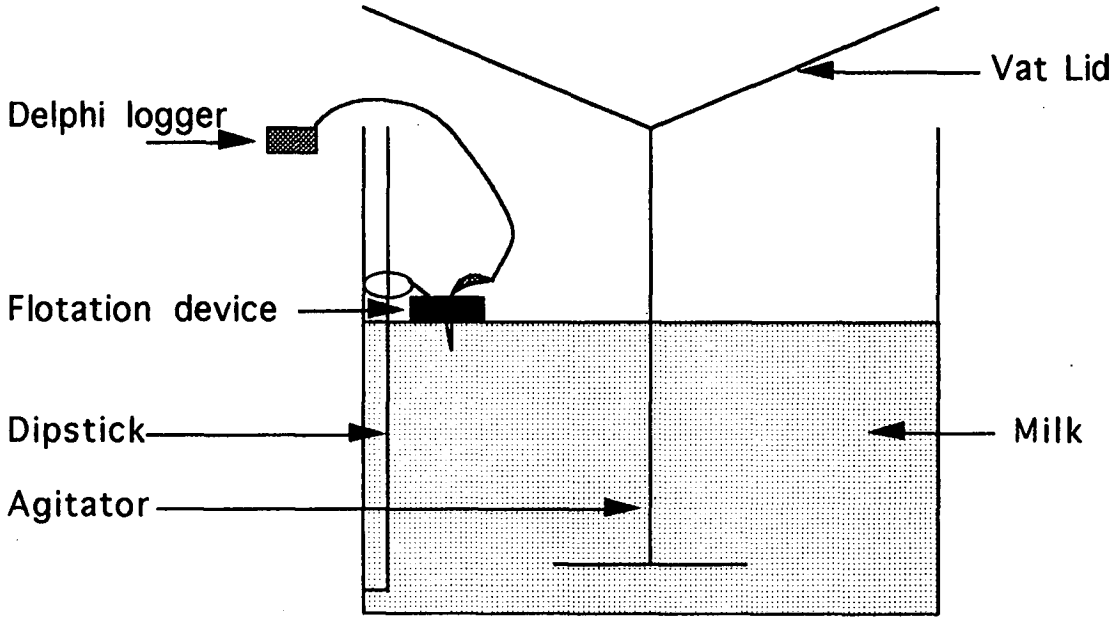
The temperature/a_w model (Equation 4.3) for pseudomonads was incorporated into a spreadsheet format, in both Lotus 123 and Microsoft Excel versions, called '*Pseudomonas* Predictor' (Ross *et al*, 1993b). '*Pseudomonas* Predictor' was designed as a template program so that other organisms and/or environmental conditons can be added in the future. '*Pseudomonas* Predictor' discs and instruction manuals were distributed to 24 'clients' (15 in industry and 9 in educational or research institutions). The industry participants included 'clients' from both dairy and meat industries. The 'clients' were asked to carry out validation trials, in which they monitored the growth of pseudomonads by viable count methods while also recording the temperature, using electronic data loggers such as the Delphi, throughout the trial time. The 'clients' then sent the viable count data together with the temperature profile to the candidate for analysis in which the temperature profile was used to determine the predicted extent of pseudomonad growth and was then compared to that observed.

3.6.1. 'Client' 1: Raw Milk

Validation studies were carried out with the co-operation of a dairy company and its supplying farmers. It was necessary to identify a site where samples could be easily removed and a reasonable amount of growth occur ie, the milk had to remain in that location for an extended length of time and/or poor cooling practices occurred. The farm milk cooling vats appeared to fit these criteria, however, prior to starting the validation trials, the company implemented a quality pays system for the milk. As a result it was difficult to find appropriate farms as they had improved to the extent that most were being paid the top price and therefore had very good quality milk. Three farms were finally chosen. The first two suspected they had cooling problems because the refrigeration units appeared to run longer than necessary, while the third farm was identified as often having trouble achieving cold temperatures at pickup and had high numbers of cells in the milk compared to the other farms.

A Delphi logger was used to monitor temperatures throughout the experiments. In order for the Delphi logger to remain at the same depth in the milk, as the level rose with each milking, and to prevent the logger from becoming entangled in the agitator, the Delphi logger was attached to a flotation device which was looped around the dipstick (Figure 3.3).

Figure 3.3 Positioning the Delphi logger in the milk cooling vats.



The flotation device is attached by a ring to the dipstick and thereby prevents the Delphi logger from sinking and becoming entangled in the agitator

While the logger was in the milk cooling vat, aliquots were removed periodically, by one of the company's microbiologists, and plated onto PSA using the procedures described in 3.4.1. The number of samples removed were restricted due to the microbiologist also having to complete their normal workload. The temperature profile was downloaded from the Delphi logger and sent to the candidate together with the pseudomonad count data for analysis, where the extent of growth observed was compared to that predicted by the model.

3.6.2. 'Client' 2: Reconstituted Whole Milk and UHT Milk

14g of milk powder was reconsituted in 90mL of water, inoculated with various strains of pseudomonads and stored at either 4 or 7°C. In one instance 200mL UHT milk in a 1L flask was inoculated and stored at 15°C in a shaking waterbath at 150rpm. Growth was monitored using a pour plate method. The growth curve data obtained by 'client' 2 was sent to the canditate where generation times were determined using methods described in 3.4.1.

3.6.3. 'Client' 3: Cream

Samples of cream were removed from a holding tank at various times and plated to obtain total plate and pseudomonad counts. The temperature of the cream was recorded at various intervals. The data obtained by 'client' 3 was sent to the canditate who determined generation times using methods described in 3.4.1. The extent of growth observed was compared to that predicted by the model from the temperature profile provided by the 'client'.

3.6.4. 'Client' 4: Minced Beef

Samples of minced beef were stored at two temperatures (2.3 and 2.2°C) and the growth of the natural biota monitored on PSA. Five trials of minced beef stored at fluctuating temperature conditions were also carried out. Temperatures, during the fluctuating temperature studies, were monitored using a Datataker and viable counts plated periodically. Data was sent to the canditate who determined generation times using methods described in 3.4.1. The extent of growth observed was compared to that predicted by the model using the temperature profile provided by the 'client'. The a_w of 12 samples of minced beef were determined by the candidate using the Aqualab CX2.

3.7. INDUSTRY RESPONSE (QUESTIONNAIRE)

At the end of the industry evaluation period 'clients' were sent a questionnaire (Appendix 2) asking them about the usefulness and user-friendliness of the software and manuals. The questionnaire was designed to contain a mixture of closed-form (e.g., questions that can be answered with 'Yes' or 'No' such as "would you use predictive models for other organisms if they were available?") or open-ended questions (e.g., Comment on "what organisms and environmental factors would you like to see models developed for?") thus providing a mixture of quantitative and qualitative information. Information on question content, wording sequence and general ideas on obtaining the best response rate were obtained from Hoinville & Jowell (1978), Warwick & Lininger (1975) and Young (1966). Questionnaires were printed on green paper accompanied by a cover letter and an addressed stamped envelope.

Page

4. RESULTS

Raw data for growth rate studies are shown in Appendices 8.3, 8.4 and 8.5, for studies in broth, in product (laboratory studies) and in product (industry studies) respectively.

4.1. PRELIMINARY STUDIES

4.1.1. Identification of Pseudomonads

Characteristics of the strains isolated are shown in Table 4.1. Those which were Gram negative, motile rods, oxidative in HL medium and oxidase positive were assigned to the genus *Pseudomonas*. Twenty of the 24 strains studied conformed to this description. Three isolates were oxidase negative motile oxidative rods tentatively identified as members of the genus *Xanthomonas* (2 isolates) and *Acinetobacter* (1 isolate) while one isolate was an oxidase negative fermentative bacterium identified as *Enterobacter cloacae*. As can be seen from Table 4.1, the characteristics of the pseudomonad isolates do not allow for easy differentiation to species level.

Differentiation within the genus *Pseudomonas* was initially on the basis of production of fluorescent pigments on Kings B medium (Lysenko, 1961; Krieg & Holt, 1984) as *Pseudomonas fragi* can be separated from other pseudomonads due to a lack of fluorescein production and 0/129 sensitivity. The degree of fluorescence between the remaining pseudomonad isolates varied with some isolates (e.g., *P. fluorescens* 1441) exhibiting strong fluorescence while others (e.g., *P. putida* 1021) were weakly positive. In two cases fluorescence was uncertain, however, this does not preclude the isolates as fluorescent pseudomonads as Lysenko (1961) noted that this may occur in some strains. Two isolates did not fluoresce and may be species other than *P. fluorescens* or *P. putida*.

In the individual tests some isolates had characteristics attributed to both *P. fluorescens* and *P. putida* e.g., *Pseudomonas* I1 has *P. fluorescens* characteristics for gelatin hydrolysis, casein hydrolysis and utilisation of trehalose and *P. putida* characteristics for utilisation of glycerol, D-xylose and m-inositol. The number of *P. fluorescens* and *P. putida* characteristics were compared for each isolate and the species with the highest number of positive characteristics allocated as the strain designation e.g., *Pseudomonas* I9 had 4 characteristics of *P. putida* and 3 characteristics of *P. fluorescens*; it was therefore designated as *P. putida*. For 4

Table 4.1 Isolate Characteristics

Isolate *‡	Source (Type of Milk)	Gram Chr	Oxidase	Ox/Ferm	Motility	King's B fluor	Gelatin hydrolysis	O/129 sens	Casein hydrolysis	Levan production	Carbohydates as a C source			
											Gycerol	D-Xylose	Trehalose	m-Inositol
Characteristics as defined by Lysenko (1961), & Krieg & Holt (1984)														
P.fluorescens		- rod	+	ox	+	+	+	r	+	+/-	-	-	+	+
P.putida		- rod	+	ox	+	+	-	r	-	-	+	+	-	-
P.fragi		- rod	+	ox	+	-	-	s	-		+/-	+/-		
Isolates under study														
(*P.putida*) 119*	whole	- rod	+	ox	+	++	++	r	+++	-	+++	-	++	+++
(*P.putida*) 1021*	whole	- rod	+	ox	+	+	++	r	+++	-	+++	-	++	+++
(*P.putida*) 1261*	low fat, high protein	- rod	+	ox	+	++	++	r	+	-	+++	+	++	+++
(*P.putida*) 1371*	low fat, high protein	- rod	+	ox	+	++	-	r	+	-	+++	+	++	+++
(*P.fluorescens*) 1412*	whole	- rod	+	ox	+	x	-	r	-	-	+	+	+	++
(*P.fluorescens*) 1441*	very low fat	- rod	+	ox	+	+++	+++	r	+++	+++	+++	++	+++	+++
(*P.putida*) 1442*	very low fat	- rod	+	ox	+	x	-	r	-	++	+++	+++	+++	-
(*P.fluorescens*) 1451*	very low fat	- rod	+	ox	+	+++	+++	r	+++	+++	+++	++	+++	++
(*P.fluorescens*) 1681*	low fat, high protein	- rod	+	ox	+	++	+++	r	++	-	+	+	+++	+++
P.fragi NCIMB 8542	-	- rod	+	ox	+	-	-	s(8mm)	-	+	++	++	++	-
(*P.fluorescens*) I1	skim at 4°C	- rod	+	ox	+	+++	+++	r	++	-	+++	+++	+++	-
(*X.maltophilia*) I2	skim at 4°C	- rod	-	ox	+	-	++	r	+++	-	-	-	-	-

(cont..)

Table 4.1 (continued)

Isolate *‡	Source (Type of milk)	Gram Reaction	Oxidase	Ox/Ferm	Motility	King's B	Gelatin hydrolysis	O/129	Casein hydrolysis	Levan production	Carbohydrate as a C source			
											Gycerol	D-Xylose	Trehalose	m-Inositol
(*A.baumannii*) I3	low fat at 4°C	- rod	-	ox	+	-	-	s(8mm)	-	+	-	-	-	-
(*X.maltophilia*) I4	skim at 4°C	- rod	-	ox	+	-	++	r	+++	-	-	-	-	-
(*P.putida*) I5	skim at 4°C	- rod	+	ox	+	+++	-	r	-	-	-	-	-	++
P.fragi I6	skim at 4°C	- rod	+	ox	+	-	-	s(8mm)	-	-	+++	+	-	-
(*P.fluorescens*) I7	skim at 4°C	- rod	+	ox	+	+++	+++	r	++	-	+++	++	+++	+++
(*P.putida*) I8.1	skim at 10°C	- rod	+	ox	+	++	+++	r	+++	+++	+++	++	+++	-
(*P.fluorescens*) I8.2	skim at 10°C	- rod	+	ox	+	++	+++	r	+++	++	+++	+++	+++	+++
(*P.fluorescens*) I9	low fat at 10°C	- rod	+	ox	+	++	-	r	-	++	+++	+++	+++	+++
(*P.fluorescens*) I10.1	skim at 10°C	- rod	+	ox	+	-	+++	r	+++	-	+++	+++	+	-
(*P.fluorescens*) I10.2	skim at 10°C	- rod	+	ox	+	+++	+++	r	+++	+++	+++	++++	+++	+++
(*P.putida*) I11	low fat at 4°C	- rod	+	ox	+	-	-	r	-	-	+	+	-	-
(*E. cloacae*) I12§	skim at 4°C	- rod	-	ferm	+		-							
S.aureus 3b	prawns	+ cocci	-	ferm	-	-	+	r	+	-	-	-	-	-
E.coli M23	-	- rod	-	ferm	+	-	-	r	-	-	+++	+++	+++	-

* = Victorian strains

‡ = Api 20NE designation shown in brackets

x = uncertain reaction

§ = isolate was ferm thus further chr were not studied

s/r = sensitive / resistant

Ox/Ferm = oxidation / fermentation

fluor = fluorescence

Chr = characteristics

X.maltophila = *Xanthomonas maltophila*

A.baumannii = *Acinetobacter baumannii*

E.cloacae = *Enterobacter cloacae*

S.aureus = *Staphylococcus aureus*

E.coli = *Escherichia coli*

for interpretation of test results see Appendix 1: Bacteriological Methods

isolates an equal number of tests were characteristic for *P. fluorescens* and *P. putida*. The species designations obtained using individual tests and API 20 NE results are compared in Table 4.2. There does not appear to be any consistency between the two methods of identification with agreement in strain designation for eight isolates and disagreement for six isolates. It was decided to use the API designation for identifying the isolates as the presumed identification can easily be replicated by other users of the strain. *P. fragi* was identified as described above due to the inability of the API system to differentiate *P. fragi* from other pseudomonads.

4.1.2. Strain Selection

Generation time (GT) data is shown in Figure 4.1. Generation times at 10.8°C ranged from 3.3 to 5.1 hours with an average GT ± SD of 4.2 ± 0.5 hours. The two fastest strains were *P. fragi* NCIMB 8542 and *P. putida* 1442, both with a generation time of 3.3 hours. *P. putida* 1442 was selected as the main isolate for modelling purposes as the origin of *P. fragi* NCIMB 8542 was unknown (S.Colombo, ATCC, USA *pers comm*), while *P. putida* 1442 was known to be of dairy origin. Other isolates were selected for modelling by choosing representatives from all three *Pseudomonas* species (*P. fluorescens*, *P. putida* and *P.fragi)* and all rates of growth, from the fastest to the slowest, to ensure that a wide selection of pseudomonad types were represented.

4.2. MODEL DEVELOPMENT

4.2.1. Temperature Models

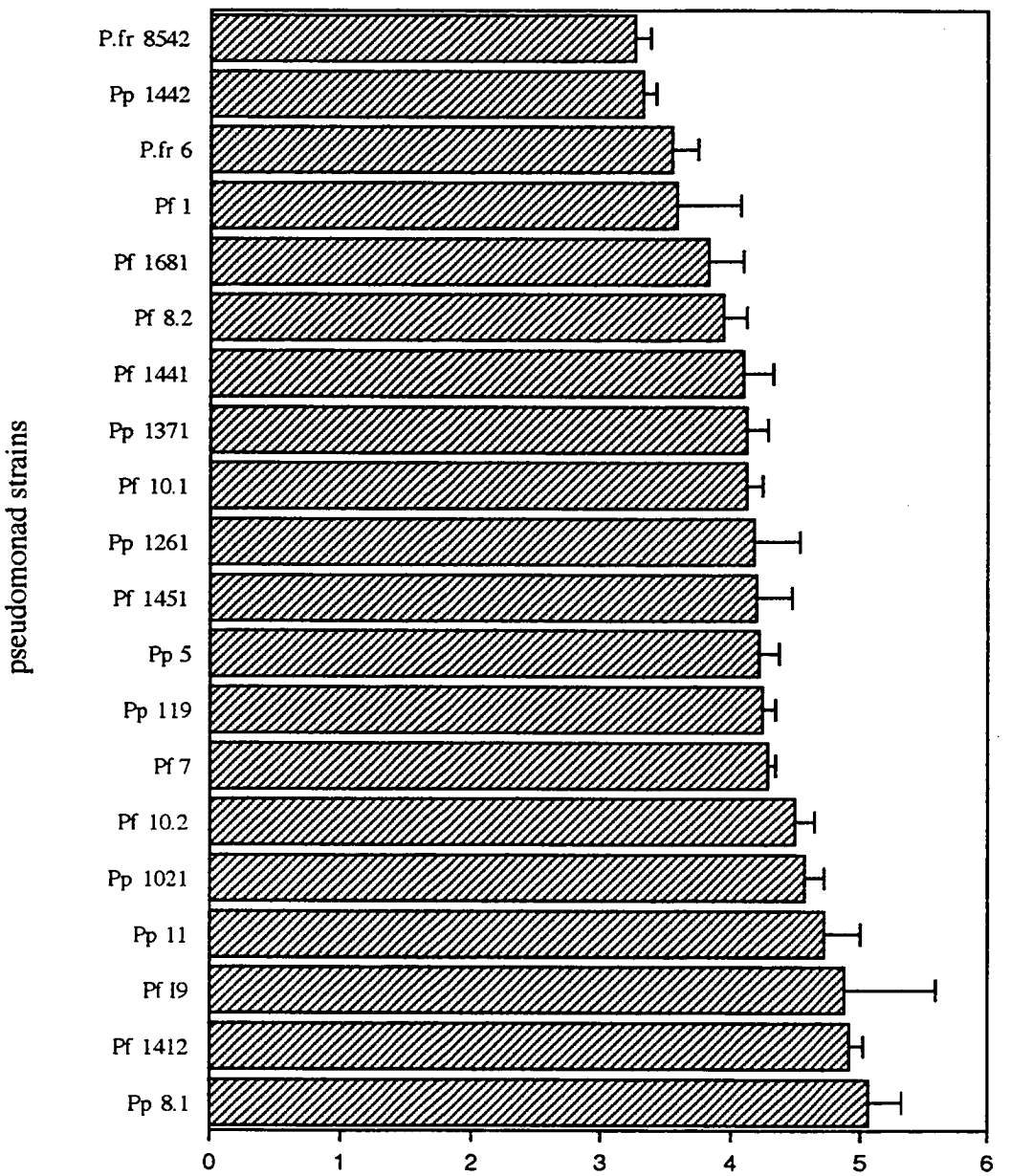
The growth rate data for *P. putida* 1442 is shown as a square root plot (Figure 4.2). Figure 4.2 combines data from two experiments, one in the temperature range 0-15°C and the other 0-30°C, and shows the high degree of repeatability of this type of experiment. As Figure 4.2 is representative of the other strains tested, the model parameters are shown in Table 4.3 rather than showing each as an individual figure. Between 26 - 30 growth curves were monitored over the temperature range 0 - 30°C resulting in high r^2 values, the lowest being the cocktail of 5 strains with an r^2 of 0.990.

It was hypothesised that the various strains of psychrotrophic pseudomonads were, in fact, members of one population. In order to test this, the 'b' values from Table 4.3 were compared using single factor ANOVA resulting in F = 0.40 (F_{crit} = 5.19) with a probability of 0.80. A similar analysis was carried out comparing the T_{min} values, of the same isolates, resulting in F = 6.32 (F_{crit} = 5.19) with a

Table 4.2 A comparison of strain designation of pseudomonads produced using API 20NE strips and the characteristic tests shown in Table 4.1. Note that *P. fragi* strains were not characterised using API 20NE as the strips do not identify *P. fragi*.

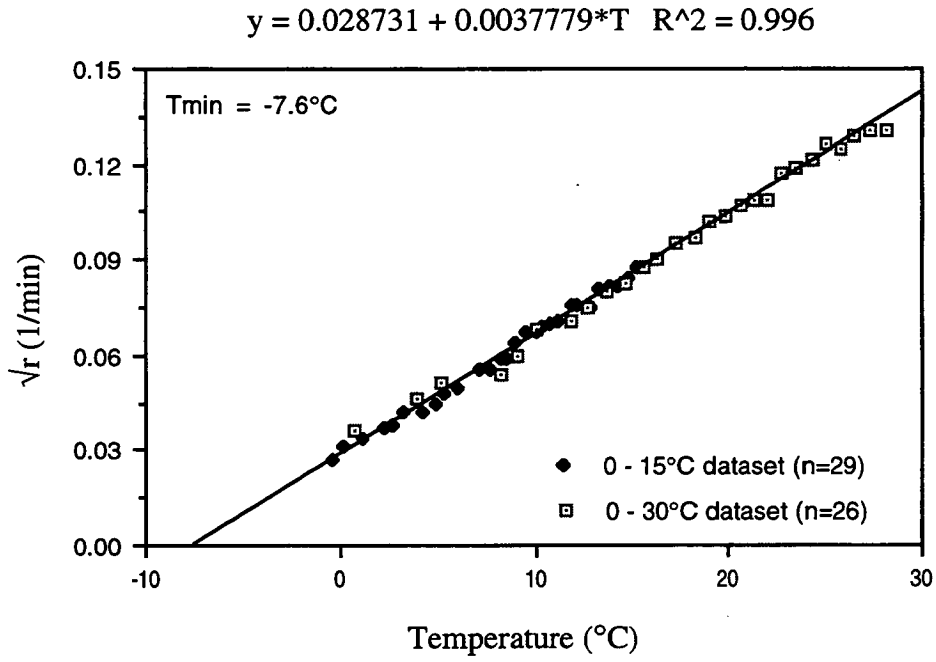
strain	API designation	% accuracy (by API 20NE)	Individual test designation	Agree / Disagree
119	*P.putida*	99.8	*P.fluorescens*	D
1021	*P.putida*	99.8	*P.fluorescens*	D
1261	*P.putida*	99.8	*P.fluorescens*	D
1371	*P.putida*	99.8	either	-
1412	*P.fluorescens*	58.5	*P.putida*	D
1441	*P.fluorescens*	81.2	*P.fluorescens*	A
1442	*P.putida*	99.8	*P.putida*	A
1451	*P.fluorescens*	81.2	*P.fluorescens*	A
1681	*P.fluorescens*	98.0	*P.fluorescens*	A
8542	-	-	*P.fragi*	-
I1	*P.fluorescens*	85.3	either	-
I5	*P.putida*	95.4	either	-
I6	-	-	*P.fragi*	-
I7	*P.fluorescens*	85.3	*P.fluorescens*	A
I8.1	*P.putida*	99.2	*P.fluorescens*	D
I8.2	*P.fluorescens*	97.5	*P.fluorescens*	A
I9	*P.fluorescens*	85.3	*P.putida*	D
I10.1	*P.fluorescens*	97.5	either	-
I10.2	*P.fluorescens*	85.3	*P.fluorescens*	A
I11	*P.putida*	64.8	*P.putida*	A

Figure 4.1 The generation time of psychrotrophic pseudomonads at 10.8°C calculated using %T data (average of 4 growth curves)



Pp = *P. putida*
Pf = *P. fluorescens*
P.fr = *P. fragi*
error bars show standard deviation

Figure 4.2 The square root plot of *P.putida* 1442 described in terms of %T.



probability of 0.034. Isolates were modelled 1-3 times, resulting in 1-3 'b' and T_{min} values for each isolate. Thus, although statistical analyses were carried out on the data, the small number of replicates means that the results must be viewed with caution. To complement the statistical analysis a visual analysis was also carried out by comparing the mean 'b' and T_{min} values in Figures 4.3 and 4.4, respectively, of the various isolates.

Given that the psychrotrophic pseudomonads belong to one population and that modelling was approached using a 'worst-case scenario', only one model (for *P. putida* 1442, the fastest strain) is needed for predicting psychrotrophic pseudomonad growth. For completeness, a model for the entire biokinetic range (Equation 1. 3) was developed using UltraFit and included data in the sub-optimal range used to derive the models for *P. putida* 1442 described in Table 4.3 and the growth rates derived from experiments in the 20 - 50°C temperature range (Figure 4.5).

Table 4.3 Model parameters for Isolates Tested in a Broth System and Described in Terms of %T.

Strain	Temperature Regime (°C)	T_{min}	b	n	r^2
P. putida 1442	0 - 30	-7.7°C (265.3K)	0.00376	26	0.993
	0 - 15	-7.4°C (265.6K)	0.00384	29	0.993
Cocktail (5 strains)	0 - 30	-6.7°C (266.3K)	0.00370	28	0.990
	0 - 15	-7.3°C (265.7K)	0.00387	30	0.990
P. fragi NCIMB 8542	0 - 30	-6.7°C (266.3K)	0.00391	26	0.997
	0 - 30	-7.3°C (265.7K)	0.00338	30	0.997
P. fragi I6	0 - 30	-5.0°C (268.0K)	0.00420	30	0.998
	0 - 30	-6.1°C (266.9K)	0.00358	30	0.992
	0 - 30	-6.1°C (266.9L)	0.00366	30	0.993
P. fluorescens 1412	0 - 30	-8.0°C (265.0K)	0.00346	28	0.993

n = number of growth curves tested to develop the square root plot r^2 = regression coefficient^2

T_{min} = notional minimum temperature for growth

Figure 4.3 Mean 'b' values of Isolates in Table 4.3

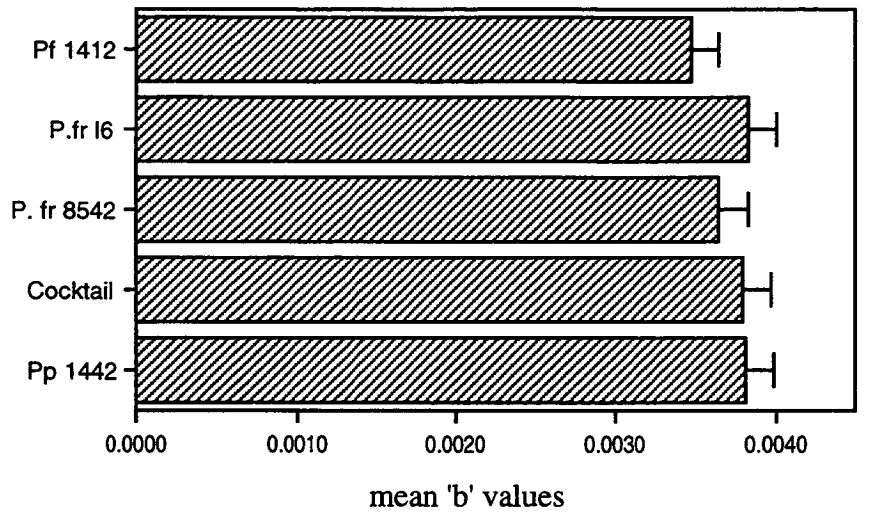
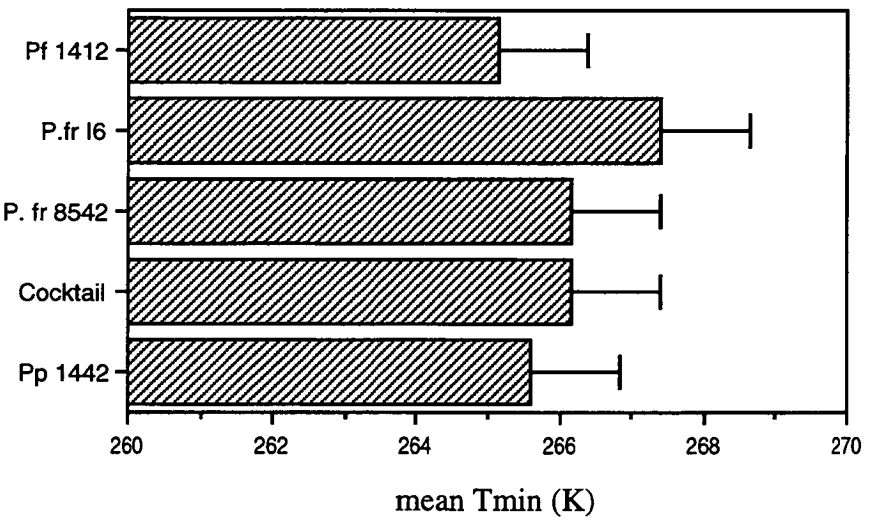


Figure 4.4 Mean T_{min} values of Isolates in Table 4.3



For Figures 4.3 and 4.4

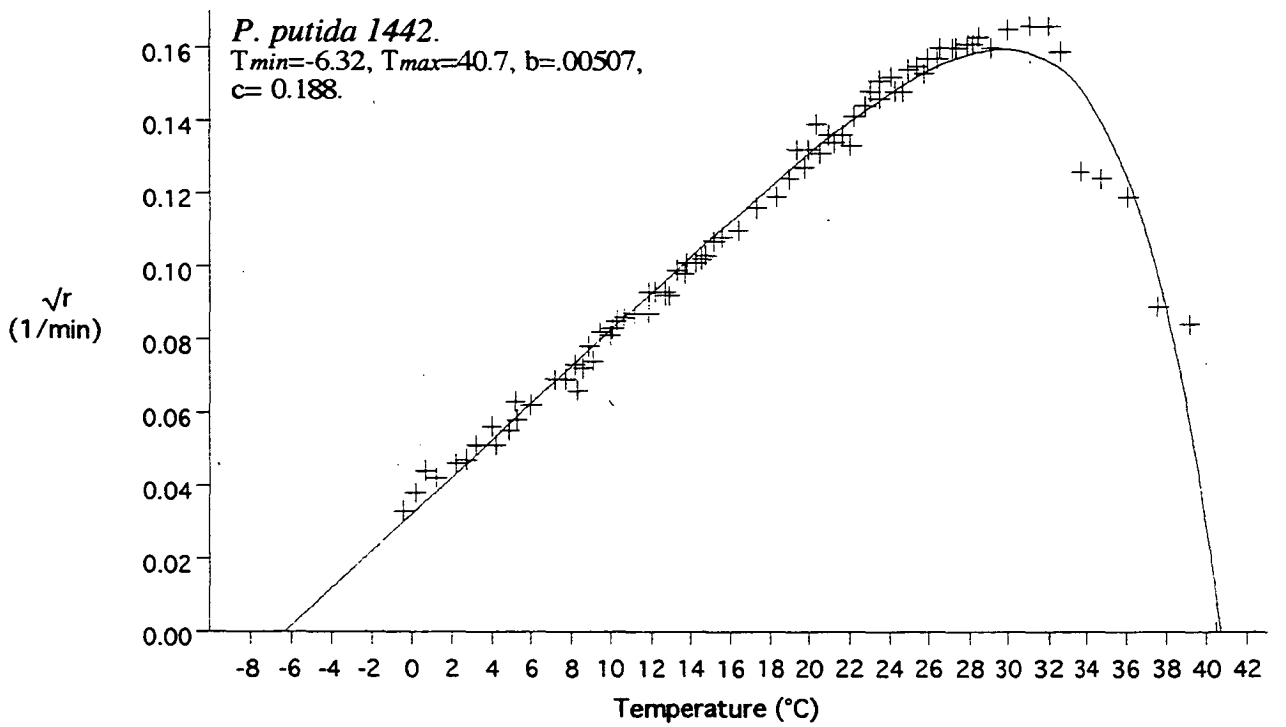
Pp = *P. putida*

Pf = *P. fluorescens*

P.fr = *P. fragi*

error bars show the pooled standard deviation

Figure 4.5 The model for *P. putida* 1442 over the entire biokinetic range (expressed in terms of VC: refer to section 4.3)



Therefore $\sqrt{r} = 0.00507(T + 6.32)\{1 - \exp [0.188(T - 40.7)]\}$

4.2.2. Water Activity Models

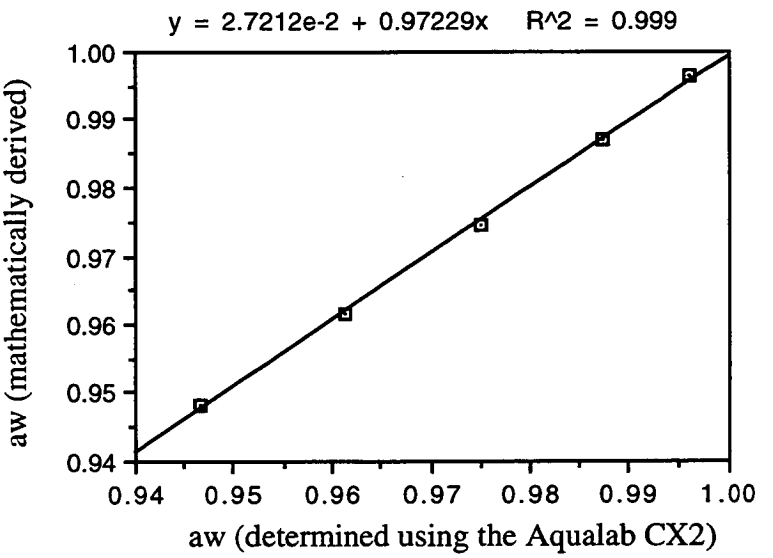
4.2.2.1. Calculation of Water Activity

Water activity measurements can be derived by a number of different methods, however, many of these are indirect methods and as such only produce comparative rather than absolute a_w measurements. The Aqualab CX2 uses the dewpoint method for a_w determination which is a primary technique and therefore provides an absolute measurement. The accuracy of the equipment was confirmed by comparing a_w derived mathematically with those observed using the Aqualab CX2. Results are shown in Figure 4.6.

4.2.2.2. Model Generation

The water activity model for *P. putida* 1442 is shown in Figure 4.7. Of interest is the upward trend observed at the lowest a_w resulting in the isolate growing below the predicted $a_{w\ min}$. This figure is representative of the models for the other strains tested. As a result, the model parameters for the other isolates are described in Table 4.4, rather than showing each as an individual figure. To test that the isolates belonged to one population ANOVA statistics were carried out on both $a_{w\ min}$ and 'b' values. For the $a_{w\ min}$ values the F statistic was 3.60, much lower than the F_{crit} of 224.6 with a probability of 0.373. A similar result was found for the 'b' values with the F statistic of 3.05 (F_{crit} of 224.6) and a probability of 0.374. A visual analysis was also carried out by comparing the 'b' and T_{min} values in Figures 4.8 and 4.9, respectively, of the various isolates.

Figure 4.6 Comparison of a_w values calculated mathematically against those determined using an Aqualab CX2.



Note: SD bars are present but cannot be seen on the graph as they were significant to the fourth decimal figure.

Figure 4.7 The water activity response for *P.putida* 1442 growth rates described in terms of %T.

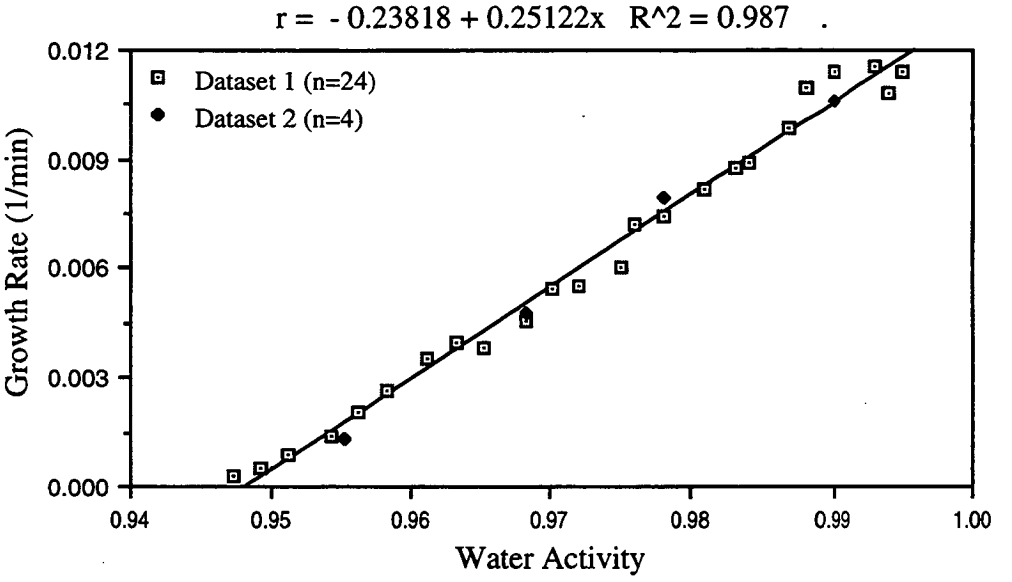


Table 4.4 Water Activity Model Parameters for Strains Tested in a Broth System and Described in Terms of %T.

Strain	$a_{w\ min}$	b	n	r^2
P. putida 1442	0.948	0.249	24	0.987
	0.950	0.271	4	0.996
P. putida 1261	0.949	0.197	16	0.992
P. fragi NCIMB 8542	0.951	0.252	14	0.992
P. fluorescens I8.2	0.952	0.250	16	0.973
P. fluorescens 1412	0.955	0.250	15	0.980
Mean:	0.951	0.245		
SD:	0.002	0.025		

n = number of growth curves tested to develop the square root plot

$a_{w\ min}$ = theoretical minimum water activity for growth

r^2 = regression coefficient^2

Figure 4.8 Mean 'b' values of Isolates in Table 4.4.

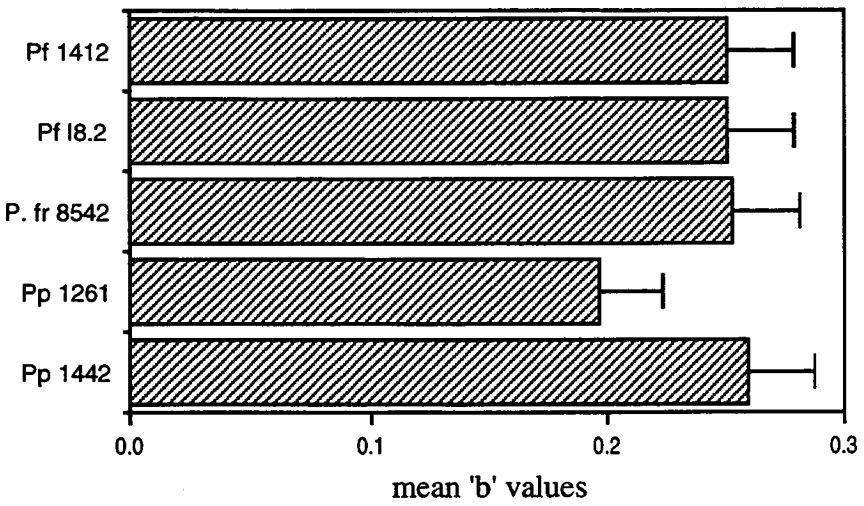
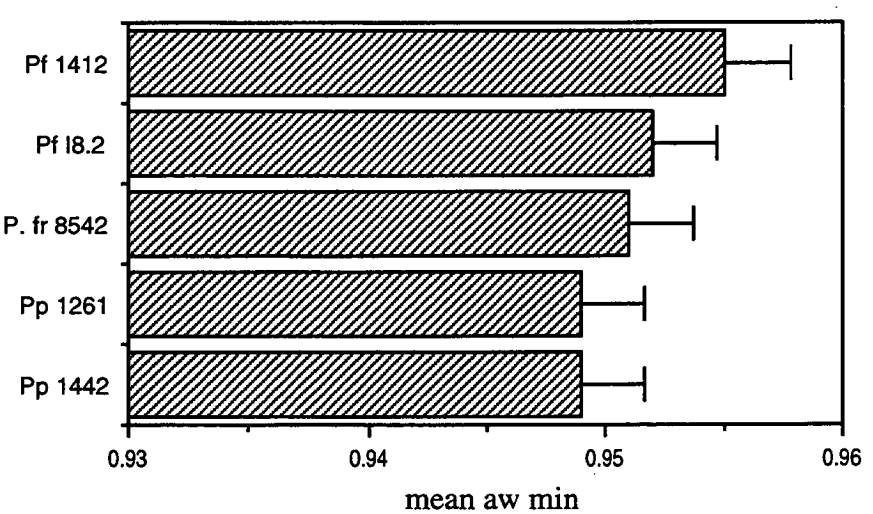


Figure 4.9 Mean $a_{w\ min}$ values of Isolates in Table 4.4.



For Figures 4.8 and 4.9

Pp = *P. putida*

Pf = *P. fluorescens*

P.fr = *P. fragi*

error bars show the pooled standard deviation

4.2.3. Combined Temperature / Water Activity Models

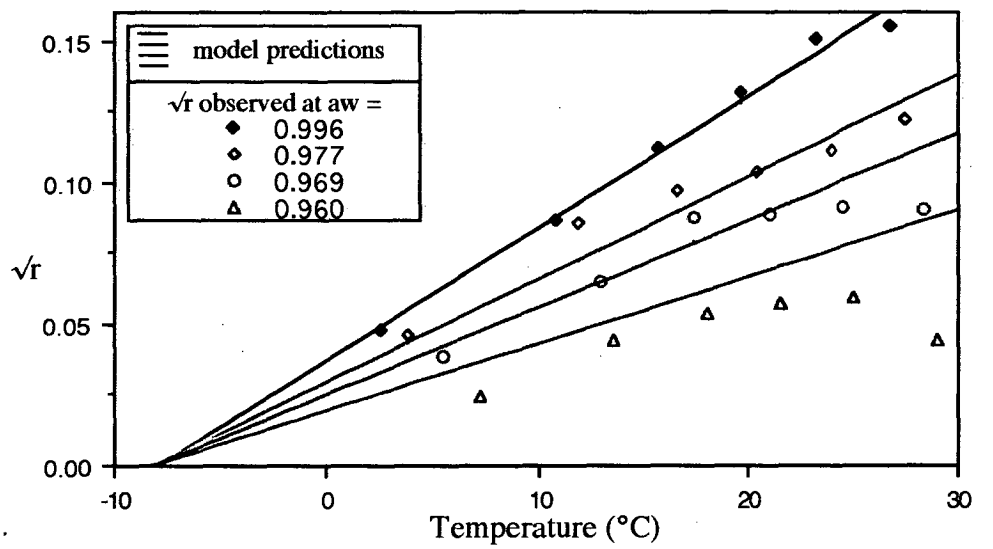
The square root plot of each strain was graphed at the various water activities and compared to the model of that strain (Figure 4.10a and 4.10b for *P. putida* 1442 and *P. fluorescens* 1412 respectively) e.g., the *P. putida* 1442 data is compared to generation times predicted by the *P. putida* 1442 model, while the *P. fluorescens* 1412 data is compared to generation times predicted by the *P. fluorescens* 1412 model. The standardised residuals ($\sqrt{r}_{predicted} - \sqrt{r}_{observed}$) were plotted against temperature (Figure 4.11a and 4.11b for *P. putida* 1442 and *P. fluorescens* 1412 respectively). The observed growth rates were compared to the temperature / a_w model for each strain using bias and accuracy factors (refer to 1.3.2.6.). Ross (1993) showed that between 20%T and 60%T there is a direct proportionality between %T and cell doubling. As this range is quite small, a final %T>20 can result in incorrect generation times, thus those cultures in which the final %T was greater than ≈20%T (i.e., Δ%T < ≈60 assuming an intial %T of ≈80) were not included in the bias and accuracy calculations. Those cultures excluded are shown in Table 4.5. Bias and accuracy factors are shown in Table 4.6.

4.2.4. Effect of pH on the Growth of Pseudomonads

The effect of pH on the growth of psychrotrophic pseudomonads at 19.1°C is shown in Figure 4.12 and shows a slight trend in growth rate, with growth rates decreasing as pH declines, between approximately pH 5.4. and pH 8. Below pH 5.4 growth rates decrease rapidly until no growth is observed at pH 5.3.

Figure 4.10 The square root plots of a) *P. putida* 1442 and b) *P. fluorescens* 1412 at various water activities, expressed in terms of %T.

a) *P. putida* 1442



b) *P. fluorescens* 1412

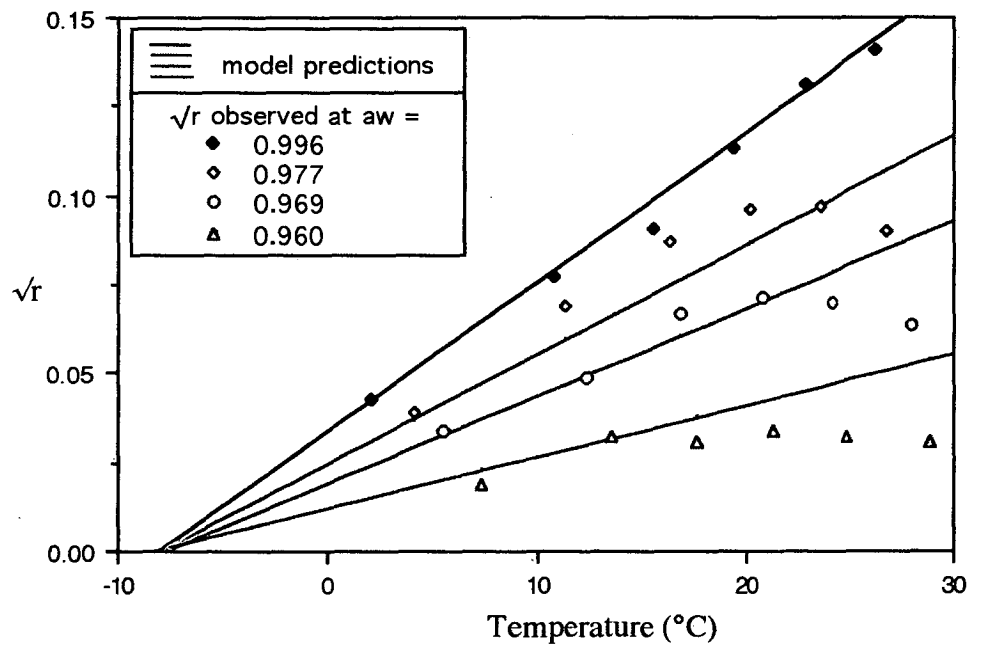
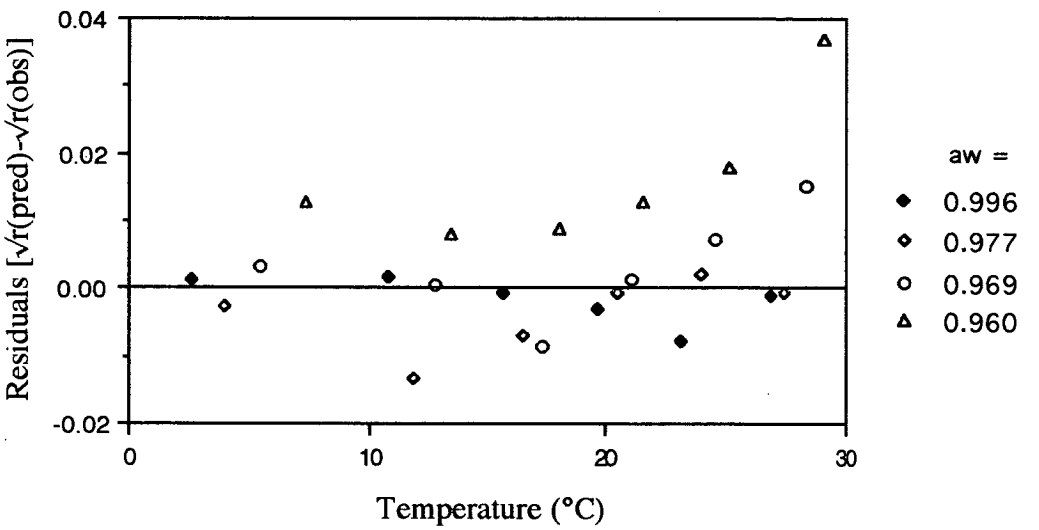


Figure 4.11 A plot of the residuals ($\sqrt{r}_{predicted}$ - $\sqrt{r}_{observed}$) versus temperature for a) *P. putida* 1442 and b) *P. fluorescens* 1412.

a) *P. putida* 1442



b) *P. fluorescens* 1412

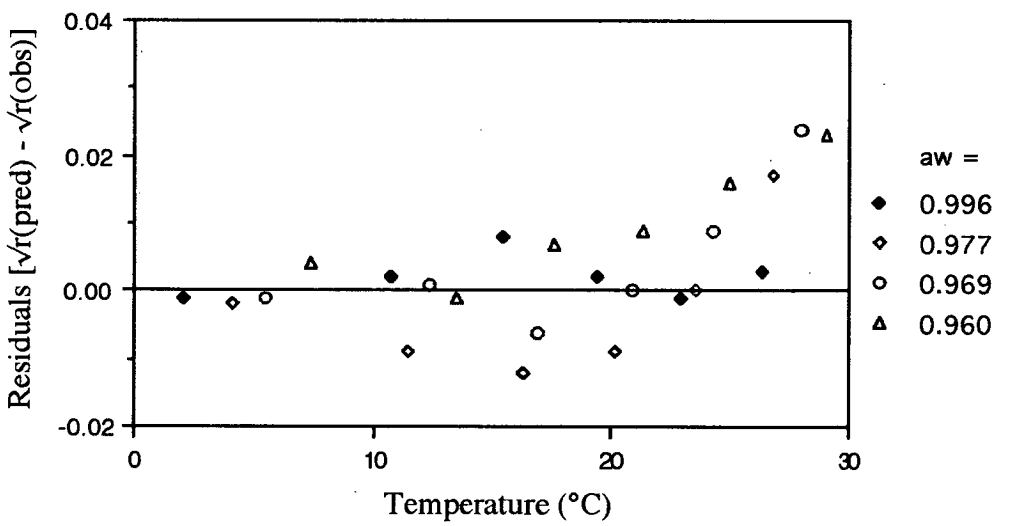


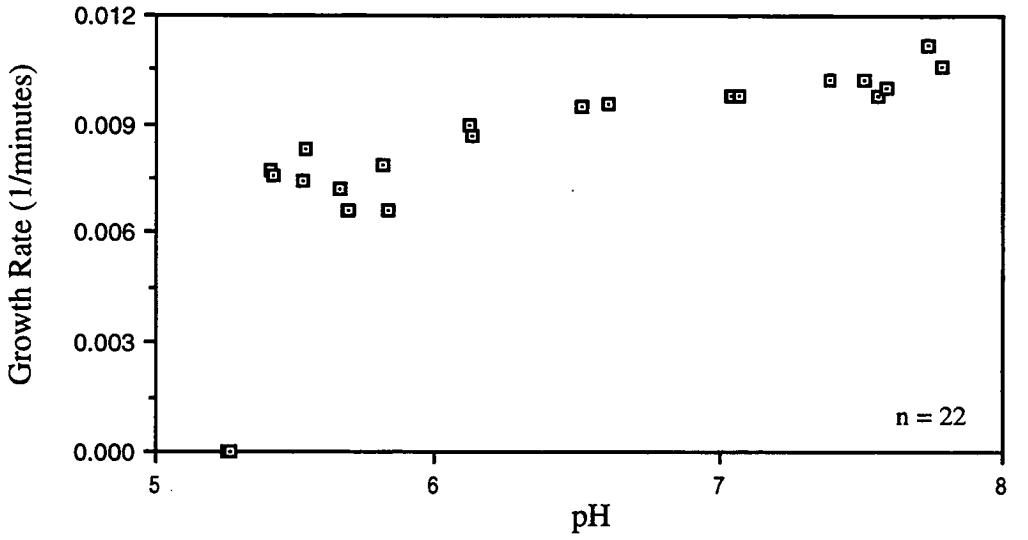
Table 4.5 The final %T values of the cultures excluded from the bias and accuracy calculations (shown in Table 4.6).

Isolate	a_w	Temperature(°C)	Final %T
P. putida 1442	0.960	7.3	51
	"	29.0	56
P. fluorescens 1412	"	7.4	31
	"	24.9	41
	"	28.0	56

Table 4.6 Bias and accuracy data comparing the growth of isolates at various water activities to the growth rates predicted by the combined a_w/temperature model for that isolate.

Isolate	a_w	Bias	Accuracy	n
P.putida 1442	0.996	0.98	1.05	6
	0.977	0.91	1.12	6
	0.969	1.08	1.16	6
	0.960	1.48	1.48	4
	Total data	1.06	1.17	22
P.fluorescens 1412	0.996	1.04	1.06	6
	0.977	0.91	1.23	6
	0.969	1.12	1.21	6
	0.960	1.32	1.32	3
	Total data	1.06	1.19	21

Figure 4.12 The effects of initial pH on the growth rate of *P. putida* 1442 at 19.1°C.



4.3. MODEL CALIBRATION

Generation times calculated by %T and VC methods are shown in Table 4.7. The ratio ($GT_{\%T}$/ GT_{VC}) was plotted against temperature (Figure 4.13). As the ratio ($GT_{\%T}$/ GT_{VC}) is constant, with respect to temperature, the generation times derived from %T methods can be divided by 1.50 (the calibration factor) and the square root model replotted to derive the new (VC) equation. T_{min} remains constant regardless of the method used to determine generation time. The 'b' values for the square root models of the isolates from Table 4.3, described in terms of both %T and VC, are shown in Table 4.8. As an example, for the combined data (n = 55) of *P. putida* 1442, the equation describing growth using turbidimetric methods is

$$\sqrt{r} = 0.028935 + 0.0037556 * T \qquad (4.1)$$

where

$\sqrt{r}$ = $\sqrt{}$growth rate (1/minutes)

T = temperature (°C)

T_{min} is -7.7°C

while the equation describing growth using VC methods becomes

$$\sqrt{r} = 0.035532 + 0.0045923 * T \qquad (4.2)$$

where

T_{min} is -7.7°C

and $\sqrt{r}$ and T are as described above

Similarly, the combined temperature and a_w model for *P. putida* 1442 over the entire biokinetic range, described in terms of VCs, is

$$\sqrt{r} = 0.1673 * \sqrt{a_w - 0.947} * (T + 7.7) * \left\{1 - \exp\left[0.192(T - 41)\right]\right\} \qquad (4.3)$$

where

$\sqrt{r}$ = $\sqrt{}$growth rate (1/hours)

T = temperature (°C)

a_w = water activity

Pseudomonads were inoculated to an intial load of 10^5 cfu/mL, however turbidimetric methods only detect changes in growth once levels are around 10^7 cfu/mL. The lag phase determined from %T data is not the true lag phase but a combination of the detection time of the spectrophotometer and the actual duration of lag phase. Therefore, once %T values start decreasing, the spectrophotometer has started to detect growth (its lower sensitivity level). This point was determined by calculating the lag phase duration (LPD) for %T data and determining the numbers of bacteria present at that time from the VC data. LPD was calculated using Equation 1.1a from parameters derived from the Gompertz function. As the LPD was often between two VC sampling times it was assumed that the difference between the samplings were linear and the cfu/mL at which the spectrophotometer started to detect growth could be calculated accordingly. This is not strictly true but provides a good approximation due to the small increase in growth observed between sampling periods. For example, at 25.6°C, the LPD was 428 minutes (Appendix 3). This time is between VC readings at 434 and 360 minutes which corresponded to 6.63 and 6.14 log cfu/mL. The difference between these are 74 minutes and 0.49 log cfu/mL, while the difference between 434 and 428 is 6 minutes which corresponds to 0.0397 log cfu/mL ([6 x 0.49]/74). Thus, in this instance, the spectrophotometer first detected growth when the viable counts were at 6.59 (6.63 - 0.0397) log cfu/mL. The lower

sensitivity limit of the spectrophotometer for *P. putida* 1442 was plotted against temperature (Figure 4.14) in order to determine whether the lower sensitivity limit of the spectrophotometer was affected by the temperature of the broth. The r^2 value of 0.119, from Figure4.14, suggests that there is no temperature effect and that the spectrophotometer has a lower sensitivity limit of log 6.9.

Table 4.7 The generation times for *P. putida* 1442 calculated by both %T and VC methods are shown, together with the ratio ($GT_{\%T}$/ GT_{VC}).

Temp (°C)	$GT_{\%T}$ (min)	GT_{VC} (min)	Ratio ($GT_{\%T}/GT_{VC}$)
25.9	55.05	38.11	1.44
25.6	55.30	36.26	1.53
21.8	70.02	51.71	1.35
21.7	74.96	47.86	1.57
17.0	115.97	78.80	1.47
16.8	106.34	65.39	1.63
12.1	171.32	97.17	1.76
12.0	168.36	100.25	1.68
5.7	291.70	228.49	1.28
5.7	277.67	210.92	1.32
4.8	508.09	345.09	1.47
4.4	542.74	345.09	1.49
mean Ratio ($GT_{\%T}/GT_{VC}$) ± SD			1.50 ± 0.15

Figure 4.13 Ratio ($GT\%T/GTVC$) versus Temperature for *P. putida* 1442.

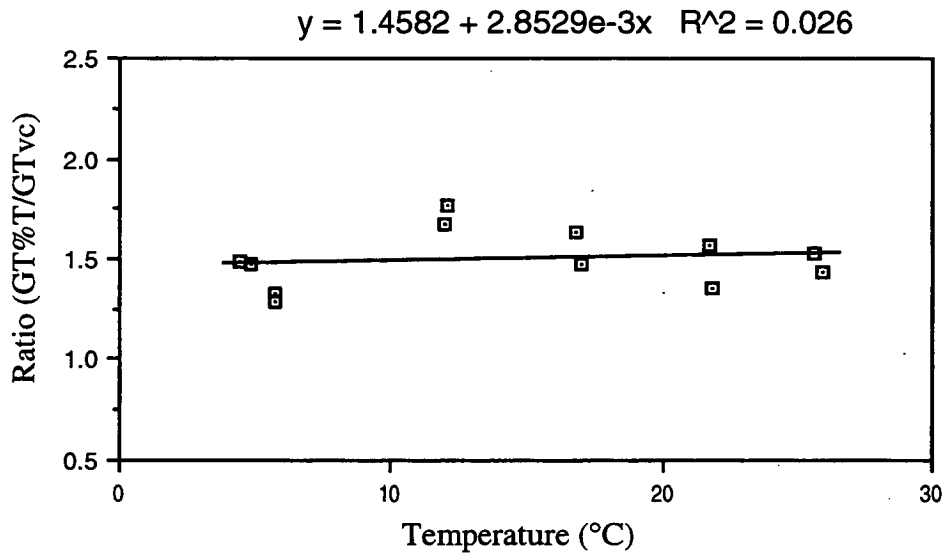
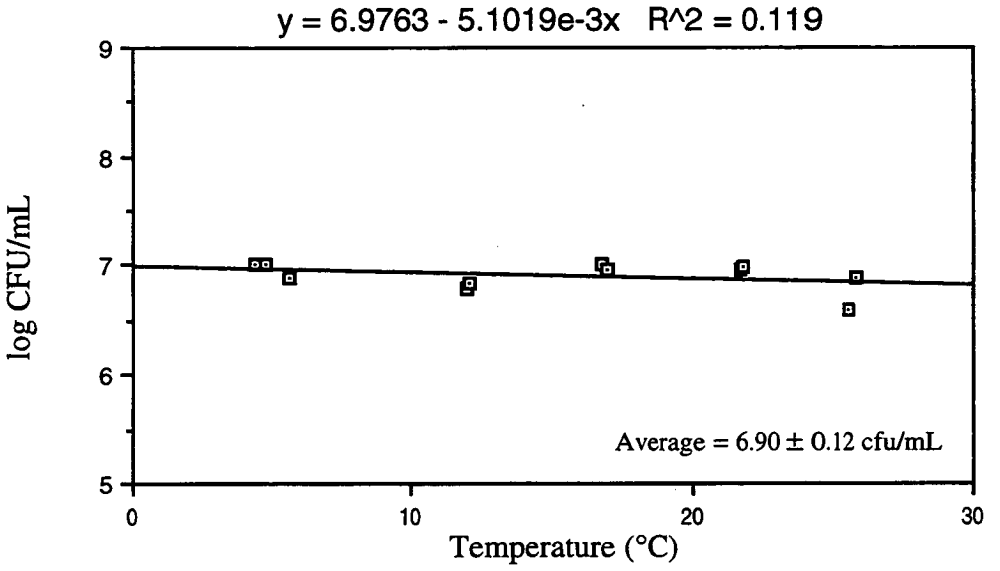


Table 4.8 'b' values for square root models of isolates tested in broth systems and under varying temperatures.

Isolate	T_{min} (°C) (from Table 4.3)	'b' in terms of %T	'b' in terms of VC
P. putida 1442	-7.7	0.0037586	0.0046013
	-7.4	0.0038377	0.0046927
Cocktail (5 strains)	-6.7	0.0037015	0.0045334
	-7.3	0.0038650	0.0047429
P. fragi NCIMB 8542	-6.7	0.0039063	0.0047851
	-7.3	0.0033825	0.0041403
P. fragi I6	-5.0	0.0042033	0.0051475
	-6.1	0.0035845	0.0043945
	-6.1	0.0036596	0.0044937
P. fluorescens 1412	-8.0	0.0034646	0.0042218

Figure 4.14 The lower sensitivity limit of the spectrophotometer for *P. putida* 1442 plotted against temperature.



4.4. MODEL VALIDATION (LABORATORY STUDIES)

4.4.1. Milk

During most of the growth curve, the pH of milk remained constant (pH = 6.68), only starting to decrease at high viable count numbers (Figure 4.15). The time to pH 6.5 was used to indicate the end of the exponential growth phase ie, the begining of the MPD plateau. When the log cfu/mL level at which pseudomonads obtain a pH of 6.5 was plotted against temperature (Figure 4.16) some temperature dependence was noticed. This effect did not decrease the utility of pH as an indicator for when viable count measurements could cease. The model parameters for pseudomonads in the various milks are shown in Table 4.9. The observed growth rates are overlayed on the square root plot of the *Pseudomonas* model (Equation 4.3) (Figure 4.17). The residuals ($GT_{predicted}$ - $GT_{observed}$) were plotted against temperature (Figure 4.18) in order to diagnose any non-linearity or non-constant error variance (Cook & Weisberg, 1982). The square root transformation was used to normalise the variance seen in Figure 4.18. Standardised residuals ($\sqrt{r}_{predicted}$ - $\sqrt{r}_{observed}$) over temperature are shown in Figure 4.19. Bias and accuracy results comparing the observed growth to that predicted by the *Pseudomonas* model (Equation 4.3) are shown in Table 4.10.

Figure 4.15 The effect on pH during the growth curve of *P. putida* 1442 in modified milk at 3.9°C.

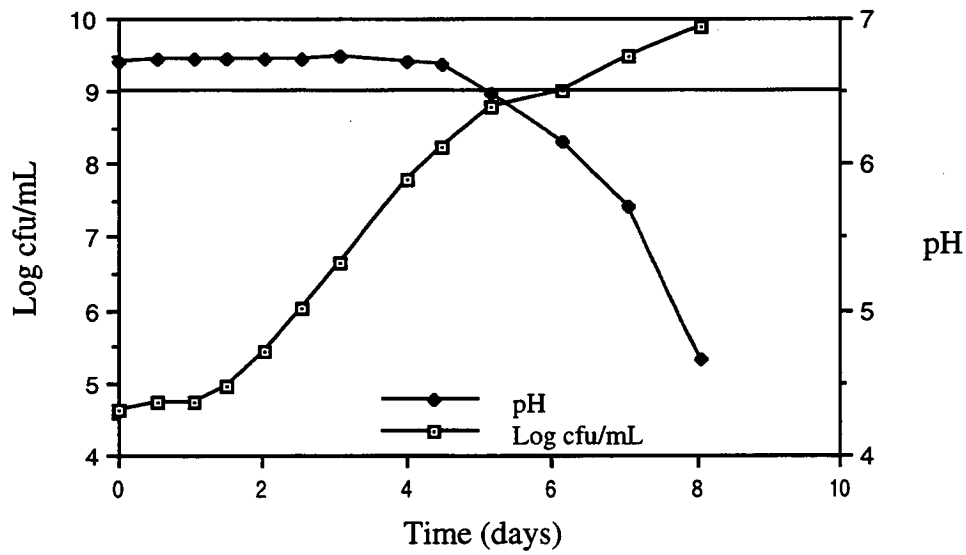


Figure 4.16 The log cfu/mL value at which pseudomonads in modified milk at various temperatures obtain a pH of 6.50.

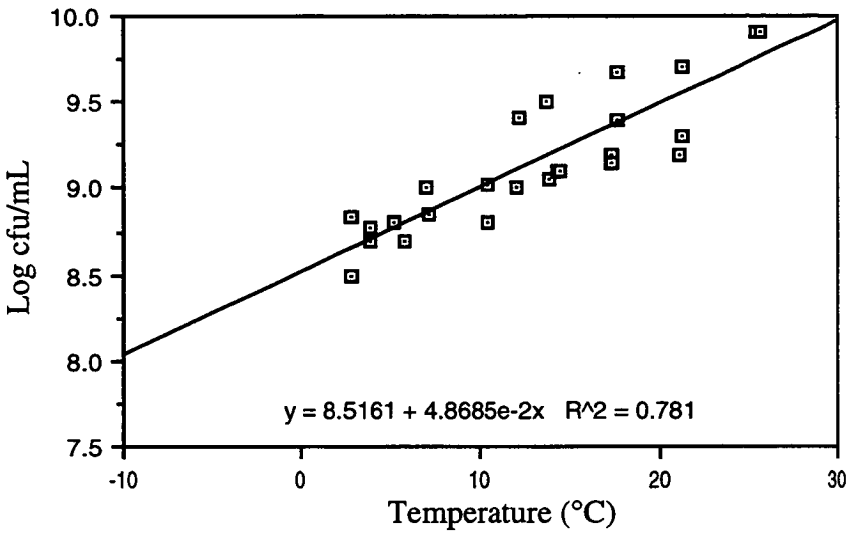


Table 4.9 Model Parameters for *Pseudomonas* in milk (laboratory studies)

Condition	Temperature Regime (°C)	T_{min}	'b'	n	r^2
P. putida 1442 in broth (from Table4.3)	0 - 30	-7.7°C (265.3K)	0.00459	56	0.996
On the TGI (ie, complete aeration)					
P. putida 1442 in modified milk	0 - 30	-7.8°C (265.2K)	0.00463	34	0.995
P. putida 1442 in whole milk	0 - 30	-7.6°C (265.4K)	0.00470	21	0.981
Uninoculated raw milk	0 - 25	-7.8°C (265.2K)	0.00449	13	0.966
TOTAL DATA		-7.7°C (265.3K)	0.00465	68	0.987
In Schott bottles (ie minimal aeration)					
P. putida 1442 in modified milk	0 - 25	-7.0°C (265.0K)	0.00444	11	0.987

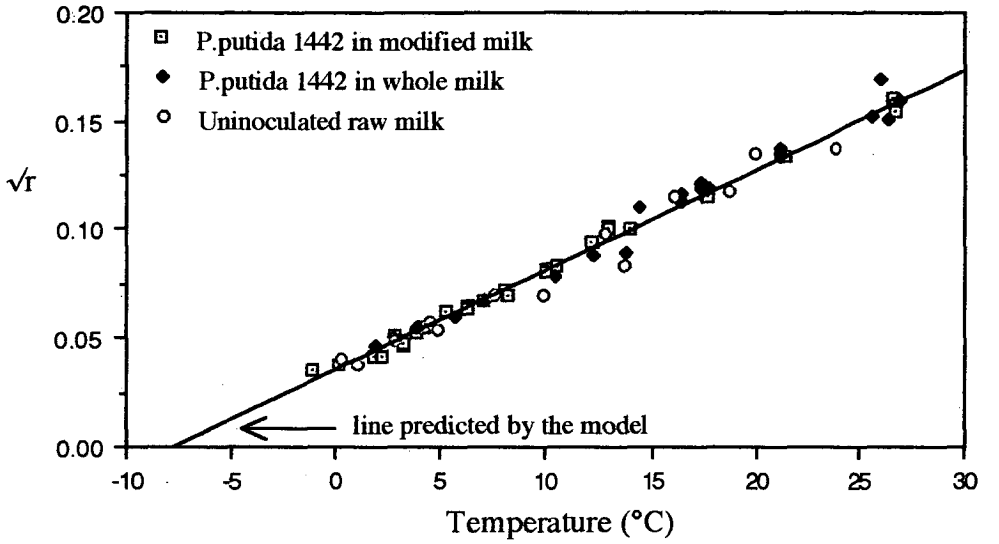
n = number of growth curves used to develop the square root plot

r^2 = regression coefficient ^{2}

'b' = parameter in square root equation

Figure 4.17 The √(growth rates) of pseudomonads in milk compared to that predicted by the model a) under aerated conditions and b) minimal aeration for VC data.
√r = √growth rate (1/min)

a)



b)

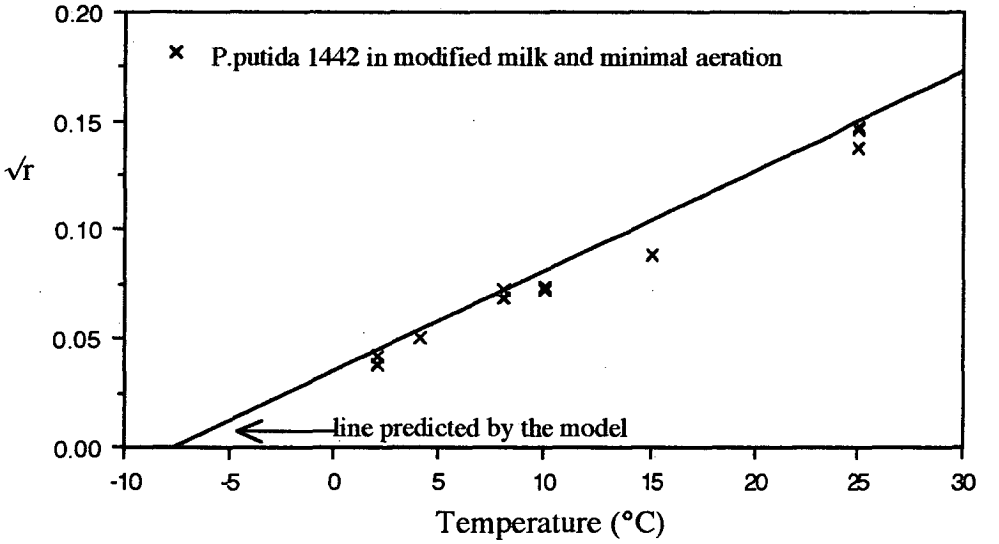


Figure 4.18 The residuals ($GT_{predicted}$ - $GT_{observed}$) versus temperature for pseudomonads in milk

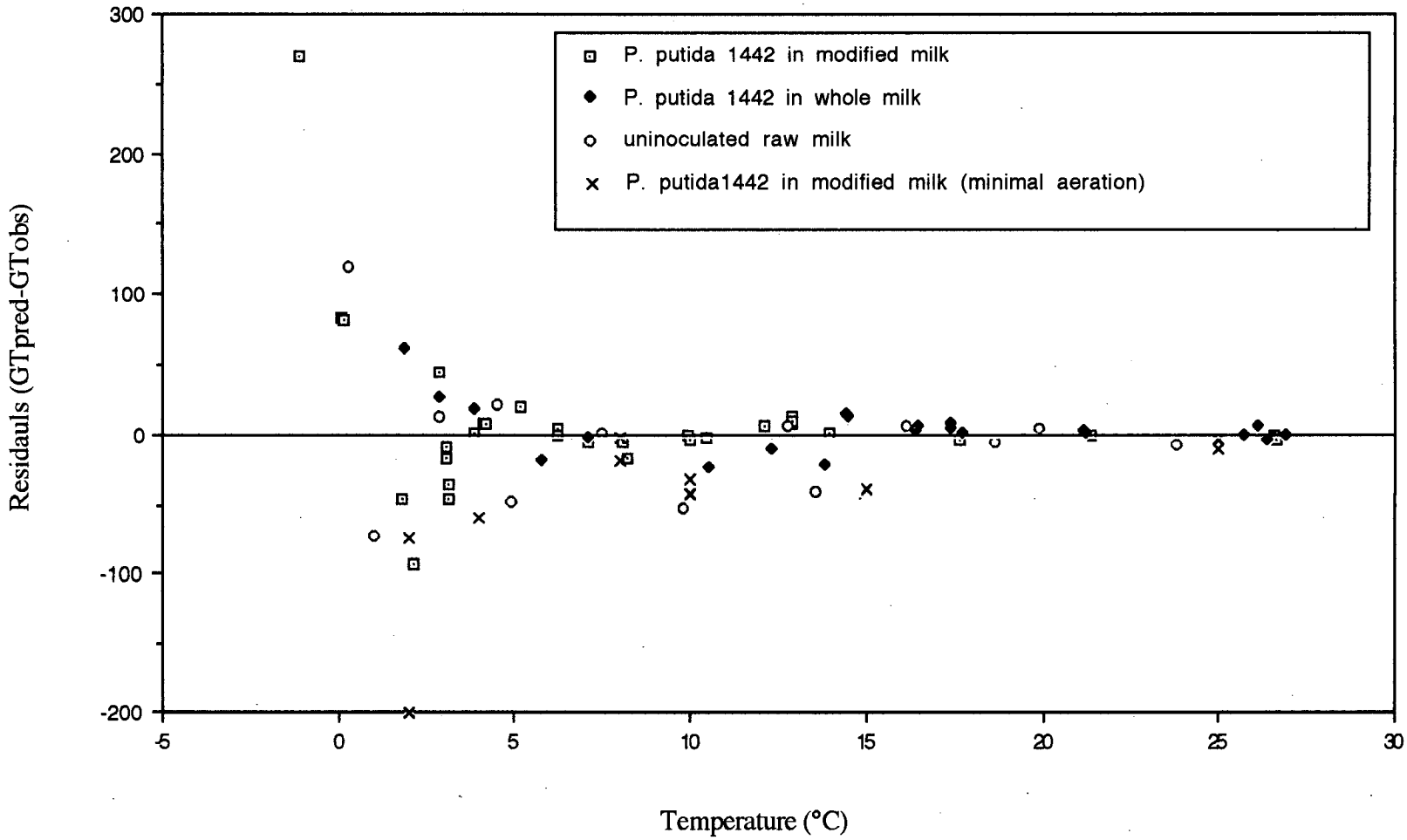


Figure 4.19 The standardised residuals ($\sqrt{r}_{predicted} - \sqrt{r}_{observed}$) versus temperature for pseudomonads in milk

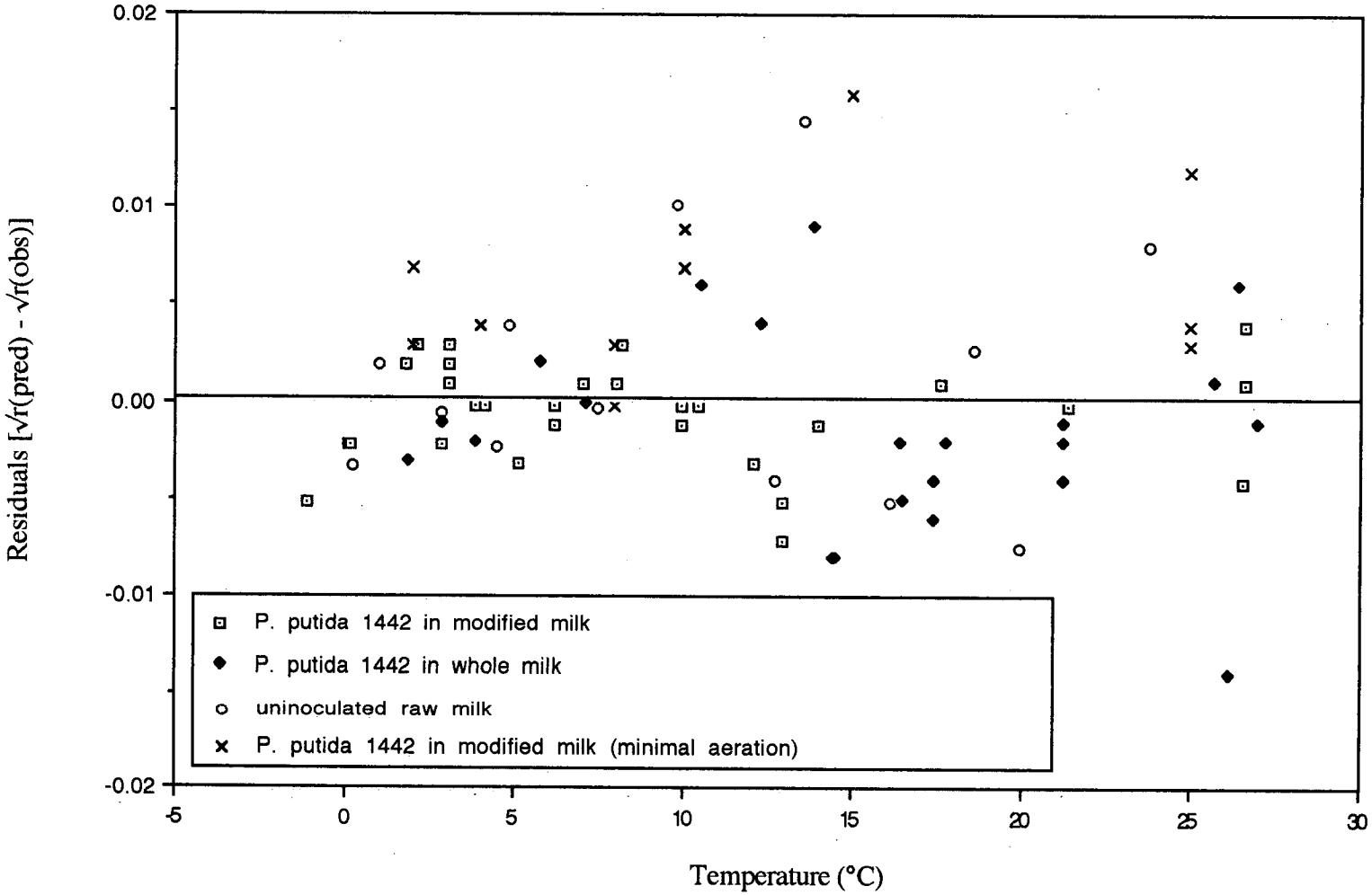


Table 4.10 Bias and accuracy values of pseudomonads in milk tested against the model for *P. putida* 1442 developed in broth.

Conditions	Bias	Accuracy	n
On the TGI (ie complete aeration)			
P.putida 1442 in modified milk	0.98	1.07	34
P.putida 1442 in whole milk	0.98	1.08	21
Uninoculated raw milk	1.03	1.13	13
Combined	0.99	1.08	68
In Schott bottles (ie minimal aeration)			
P.putida 1442 in modified milk	1.17	1.17	11

4.4.2. Evaporated Milk

The water activity of evaporated milk was measured as 0.987 ± 0.001 (n=5). Both the PCA and PSA generated data were plotted against temperature in the form of a square root plot (Figure 4.20). At high temperatures (above 19°C) growth rates calculated from PCA and PSA differed suggesting that non-pseudomonad contaminants were dominant. To resolve this problem the growth curves for *P. putida* 1442 in evaporated milk plated on both PCA and PSA, at 25.9 and 2.8°C respectively, are shown in Figure 4.21. As expected at 2.8°C the growth curves on PSA and PCA are identical. At 25.9°C the growth rates differed. The standardised residuals ($\sqrt{r}_{predicted} - \sqrt{r}_{observed}$) and normal residuals ($GT_{predicted} - GT_{observed}$) were plotted against temperature (Figure 4.22a,b), although only PCA data less than 19°C was used. In instances where generation times calculated from both PCA and PSA data were available only the PSA-calculated result was used. Bias and accuracy factors are shown in Table 4.11.

Figure 4.20 Square root plot of the growth of *P.putida* 1442 in evaporated milk

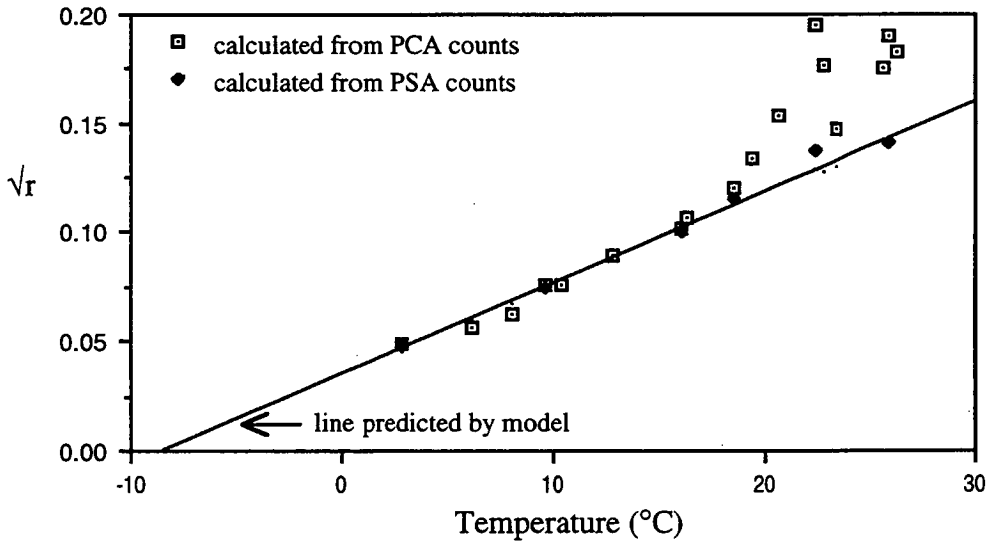
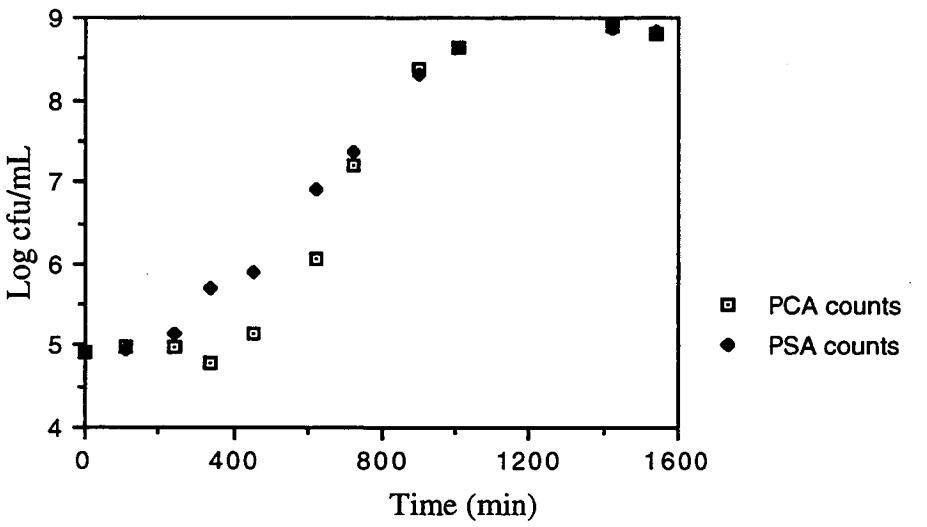


Figure 4.21: Growth curves of *P.putida* 1442 in evaporated milk and plated onto PCA and PSA.

a) at 25.9°C



b) at 2.8°C

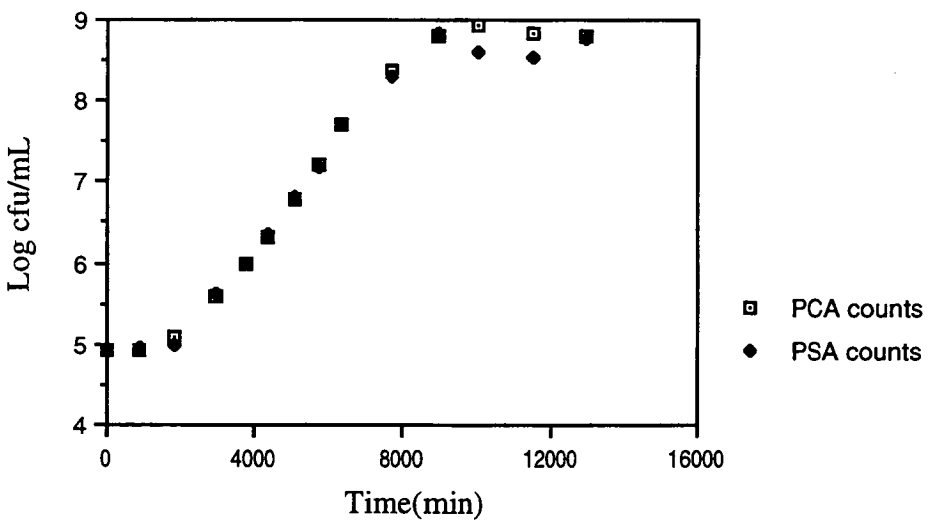
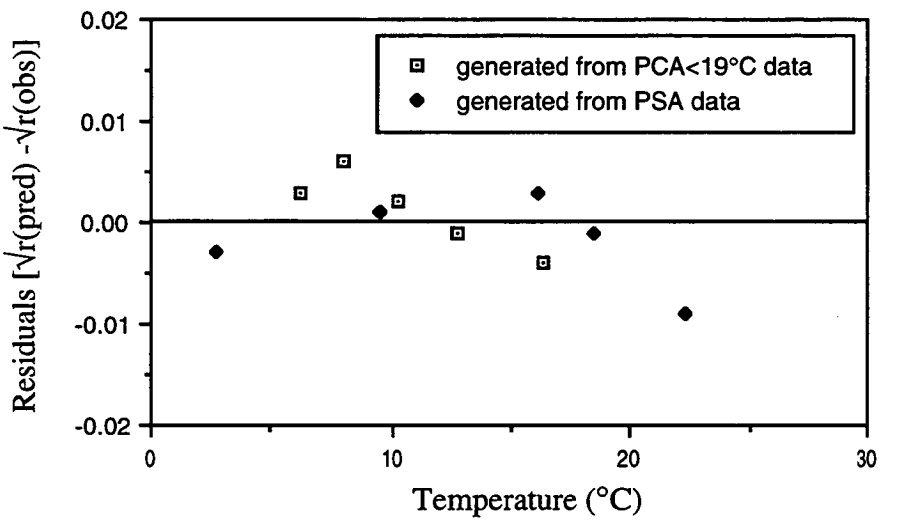


Figure 4.22 A plot of a) standardised residuals ($\sqrt{r}_{predicted}$ - $\sqrt{r}_{observed}$) and b) normal residuals ($GT_{predicted}$ - $GT_{observed}$) versus temperature for *P. putida* 1442 in evaporated milk.

a) standardised residuals ($\sqrt{r}_{predicted}$ - $\sqrt{r}_{observed}$)



b) normal residuals ($GT_{predicted}$ - $GT_{observed}$)

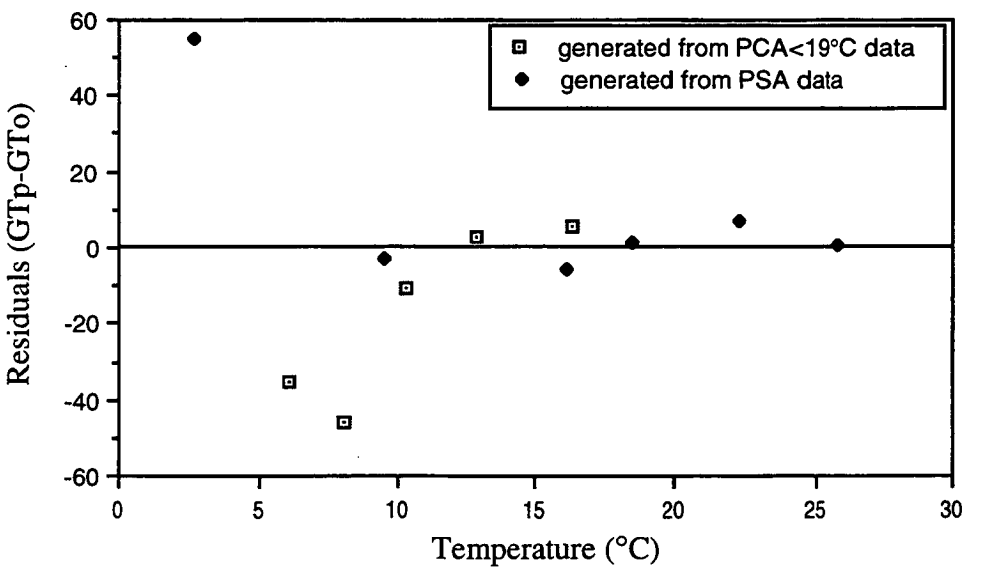


Table 4.11: Bias and accuracy factors comparing the growth of *P. putida* 1442 in evaporated milk to the model.

	Bias	Accuracy	n
1. PSA data only	0.97	1.06	6
2. PCA data at temperatures <19°C	1.06	1.10	5
3. Data from 1 & 2 combined	1.00	1.08	11

4.4.3. Cream

For both brands of cream the a_w was 0.995 ± 0.001 (n=5). Figure 4.23 shows the growth curve of the natural biota in brand 2 (both pseudomonad and total viable counts) at 11°C. Above 15 °C pseudomonads were no longer the dominant flora and were not detected on PSA at the dilutions, plated. Figure 4.24 shows the square root plot of both brands of cream (in brand 2, generation times were calculated using PSA data only), while Figure 4.25 is a plot of the standardised residuals ($\sqrt{r}_{predicted} - \sqrt{r}_{observed}$) against temperature. Bias and accuracy factors are shown in Table 4.12.

Figure 4.23 Growth curves of the natural microbiota of cream (brand 2) at 11°C.

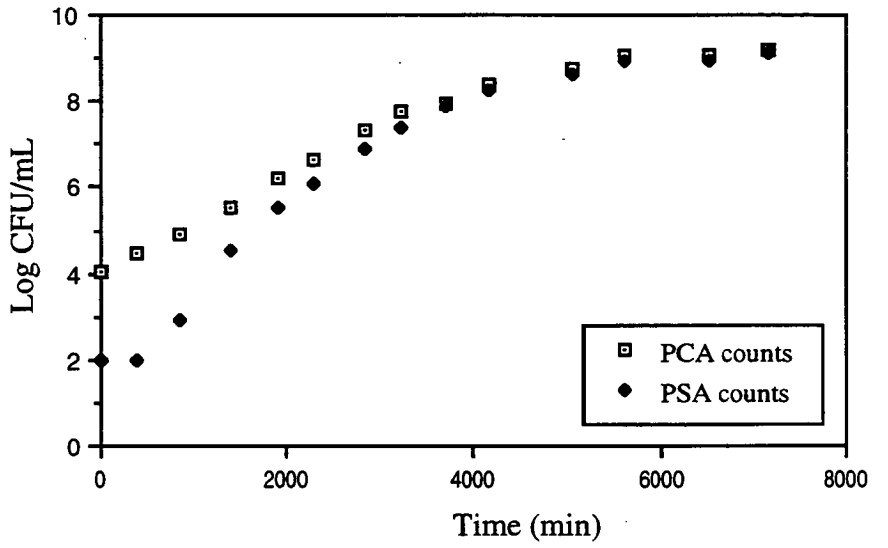


Figure 4.24 Square root plot of the growth of pseudomonads in cream.

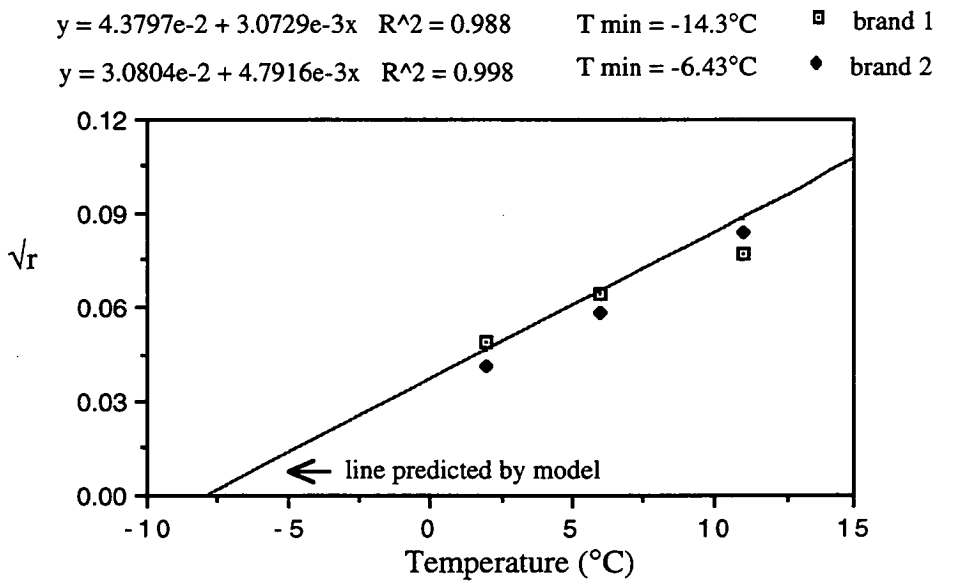


Figure 4.25 The standardised residuals ($\sqrt{r}_{predicted}$ - $\sqrt{r}_{observed}$) versus temperature for pseudomonads in cream.

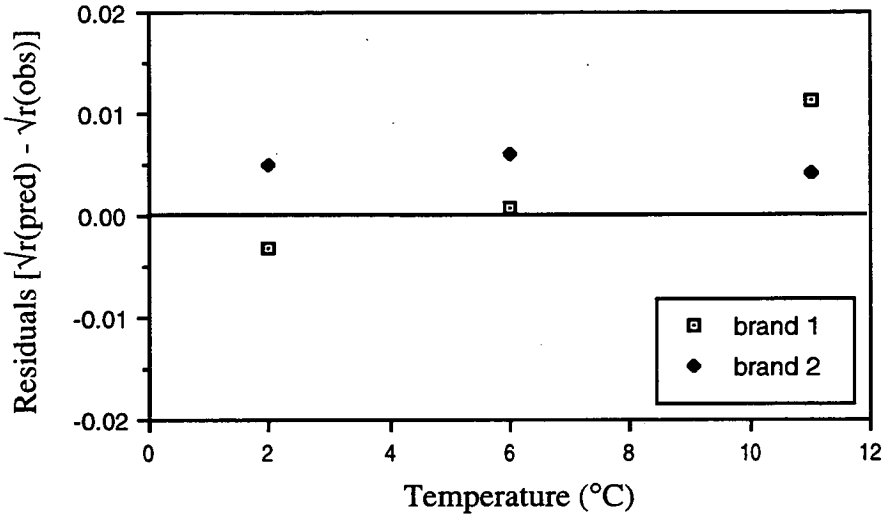


Table 4.12 Bias and accuracy factors comparing the growth of pseudmonads in cream to the model (Equation 4.3).

	Bias	Accuracy	n
1. Brand 1	1.06	1.15	3
2. Brand 2	1.19	1.19	3
3. Data from 1 & 2 combined	1.12	1.17	6

4.5. MODEL VALIDATION (INDUSTRY STUDIES)

The extent of growth predicted for each trial was calculated using the *Pseudomonas* model and the temperature history incorporated into prototype software, called '*Pseduomonas* Predictor' (Ross *et al*, 1993b). An example of the graphs produced by the '*Pseduomonas* Predictor' is shown in Figure 4.26. Raw data for industry validation studies are shown in Appendix 8.5.

4.5.1. 'Client'1: Raw Milk

A summary of the predicted and observed extent of growth for 'client' 1 is shown in Table 4.13. In Trial 1 (of Farms 1 and 2) no colonies were detected on the pseudomonad plates. This was due to incorrect dilutions being plated rather than no pseudomonads present. Similarly, in Trial 2 (of Farms 1 and 2), although colony forming units were counted, they were not present in statistically valid numbers ie, less than 30 colonies per plate. Farm 3 provided statistically valid data.

Farms 1 and 2 took between 5 and 6 hours to cool the milk to 4°C. Farm 3, which previously had problems cooling its milk, achieved 4°C within one hour. The temperature in the vat of Farm 3 was monitored for approximately 30 hours resulting in 1.9 and 1.4 logs of growth (ie, 6.3 and 4.7 generations) being predicted for Trials 1 and 2 respectively. These predictions far exceeded the observed growth. A plot of pseudomonad counts against time of Farm 3, Trial 1, Second Milking (Figure 4.27) shows that a very long lag phase exists. Unfortunately, no samples were plated between 10 and 18 hours to verify the slope of the active growth period. Similar lag periods were also observed during the other raw milk trials. If only the period of active growth is utilised, and trials with no colonies on the plates discounted, only Farm 1 Trial 2 and Farm 3 Trial 1 can be used. Revised predicted and observed extents of growth are shown in Table 4.14.

Figure 4.26 An example of an graph produced by '*Pseudomonas* Predictor'

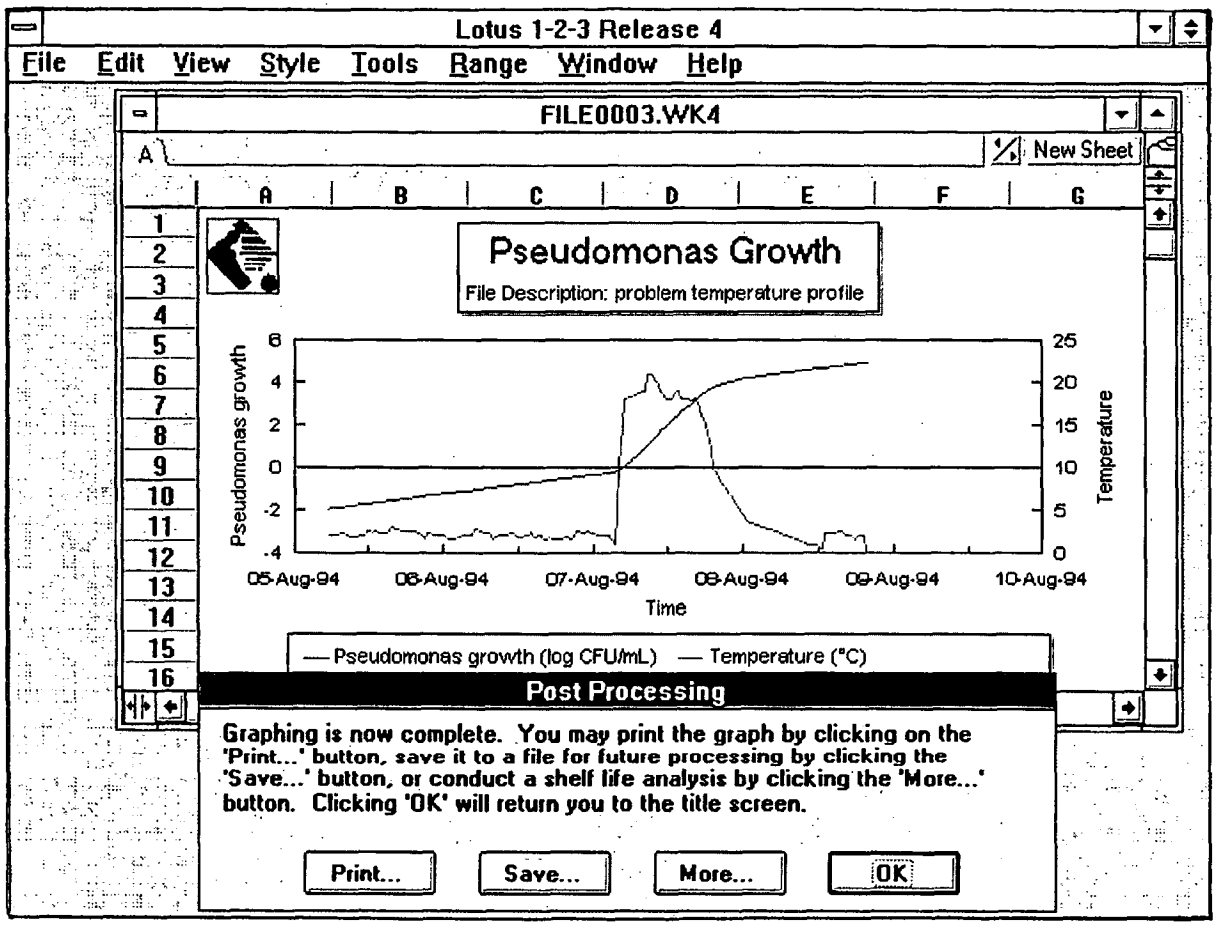


Table 4.13 The observed and predicted extent of growth for each farm trial.

Sample	No. of milkings	time (hours)	predicted extent of growth (logs)	observed extent of growth (logs)
Farm 1				
- Trial 1	2	12:00	0.68	nc
- Trial 2	2	12:02	0.80	0.68 (nsv)
Farm 2				
- Trial 1	1	3:58	0.65	nc
- Trial 2	1	2:49	0.50	0 (nsv)
Farm 3				
- Trial 1	3	31:41	1.9	0.96
- Trial 2	2	29:29	1.4	0.07

nc = no colonies on dilutions plated

nsv = not statistically valid ie, <30 colonies per plate.

Figure 4.27 Pseudomonad counts versus time for Farm 1, Trial 1, second milking period

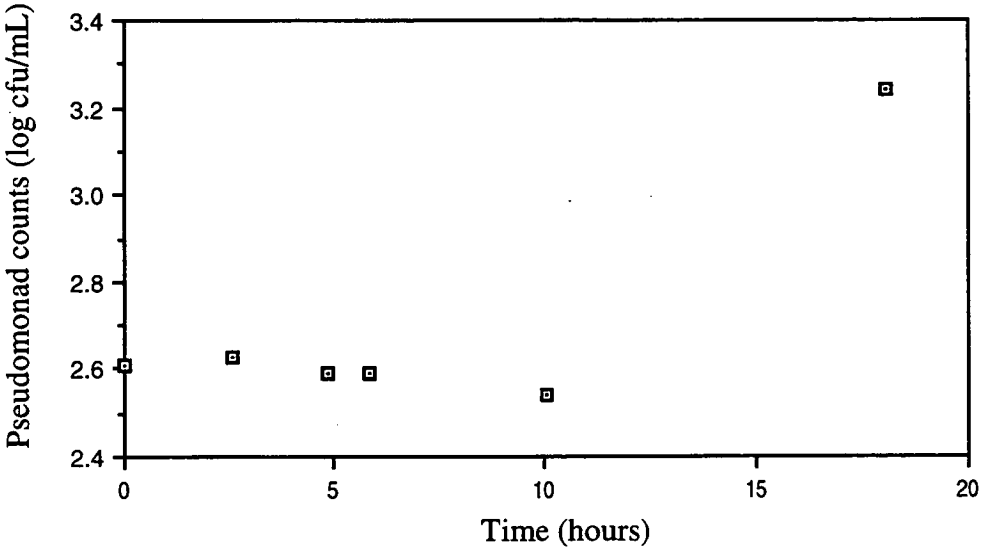


Table 4.14 Revised predicted and observed extents of growth using only data from actively growing periods.

Sample	Time (hours)	predicted extent of growth (logs)	observed
Farm 1 Trial 2	12:02	0.80	0.68 (nsv)
Farm 3 Trial 1	15:40	1.04	0.97
	Bias	1.12	
	Precision	1.12	

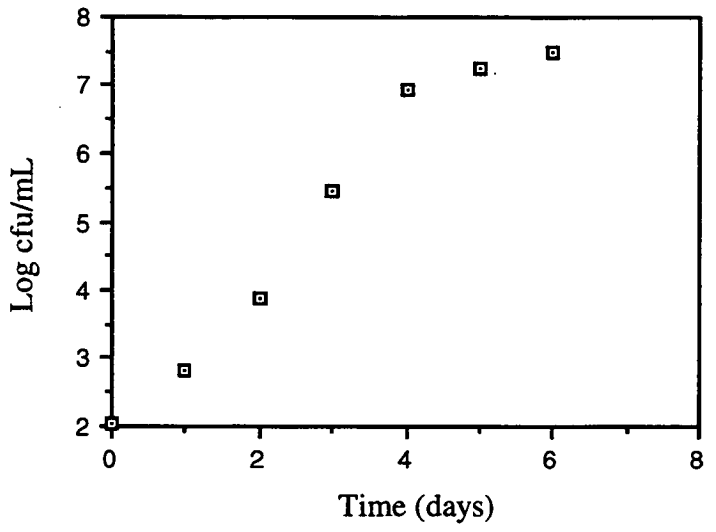
4.5.2. 'Client' 2: Reconstituted Whole Milk and UHT Milk

Observed and predicted generation times, together with the bias and accuracy factors, for the data from 'client' 2 are shown in Table 4.15. An example of the growth curves provided by 'client' 2 is shown in Figure 4.28.

Table 4.15 Observed and predicted generation time data ('client' 2).

Food Type	Temperature (°C)	Generation Times (min) Predicted	Observed
UHT Milk	15	84.6	111.0
	BIAS:	1.31	
	ACCURACY:	1.31	
Reconstituted Whole Milk	7	199.2	298.6
			263.9
			254.6
			178.9
			265.4
Reconstituted Whole Milk	4	313.8	348.1
			340.3
			332.2
			235.5
			304.0
	BIAS:	1.11	
	ACCURACY:	1.21	

Figure 4.28 An example of the growth curves obtained from 'client' 2. This particular growth curve is a pseudomonad strain inoculated into reconsituted whole milk at 7°C.



4.5.3. 'Client' 3: Cream

The data supplied by 'client' 3 suggested that there was an obvious lag phase, however there was insufficient data to accurately determine its duration. A lag of 2000 minutes was assumed for the purposes of plotting the predicted curve together with the total and pseudomonad counts (Figure 4.29). Bias and accuracy were calculated using observed points marked 1-6 on Figure 4.29 and the predicted line assuming a lag phase of 2000 minutes duration (Table 4.16).

Figure 4.29 Total and pseudomonad counts of cream ('client' 3).

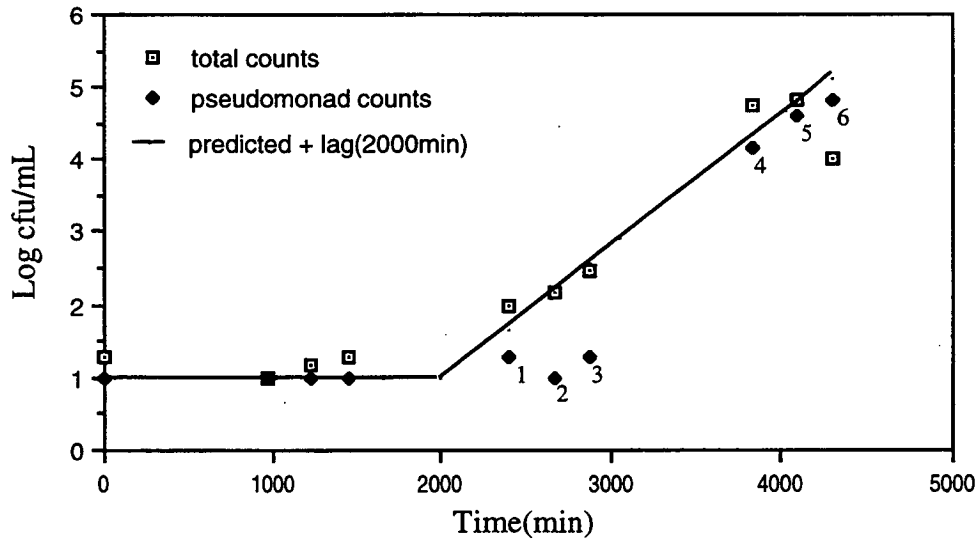


Table 4.16 Observed and predicted generation times calculated from points 1-6 on Figure 4.29.

Time Period	Times (min)	
	Observed	Predicted
1	2400	2147
2	2667	2000
3	2880	2147
4	3840	3733
5	4093	3973
6	4320	4107
BIAS:	1.14	
ACCURACY:	1.14	

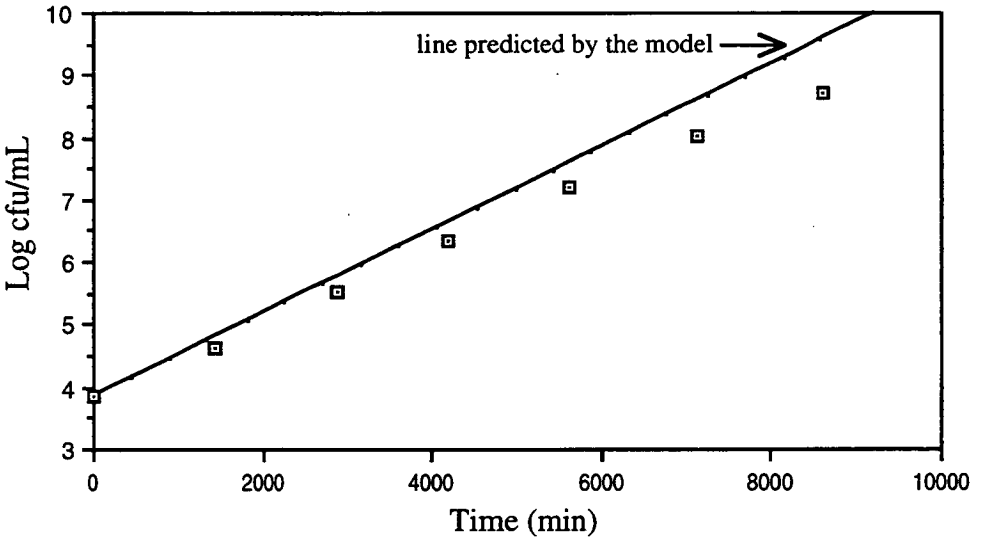
4.5.4. 'Client' 4: Minced Beef

The growth curves at 2.2 and 2.3 °C provided by 'client' 4 are shown in Figure 4.30. In both instances the lack of data prohibited using the Gompertz function to calculate generation times. As a result, generation times were calculated manually by determining the time taken for numbers to increase by 0.301 log cycles (Ross, 1993) over the steepest part of the growth curve. Both bias and accuracy factors had values of 1.06 (n=2).

The a_w of minced beef was 0.995 ± 0.001 (n=12). Figure 4.31 shows the observed viable counts, for the five trials, and the growth curves predicted by the model under fluctuating temperature regimes. Bias and accuracy factors for minced beef under fluctuating temperature conditions are shown in Table 4.17.

Figure 4.30 The growth curves obtained from client 4 showing the growth of pseudomonads in minced beef at a) 2.2°C and b) 2.3°C.

a)



b)

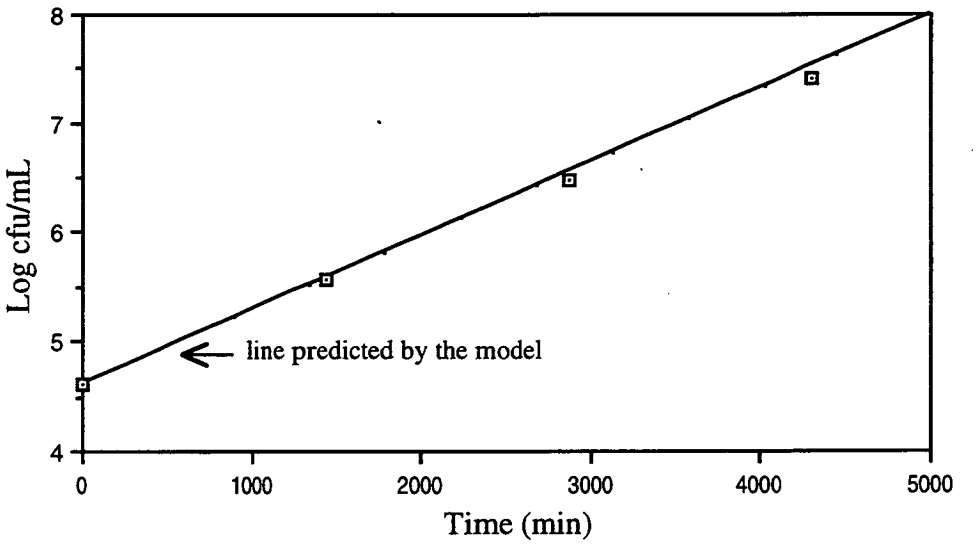
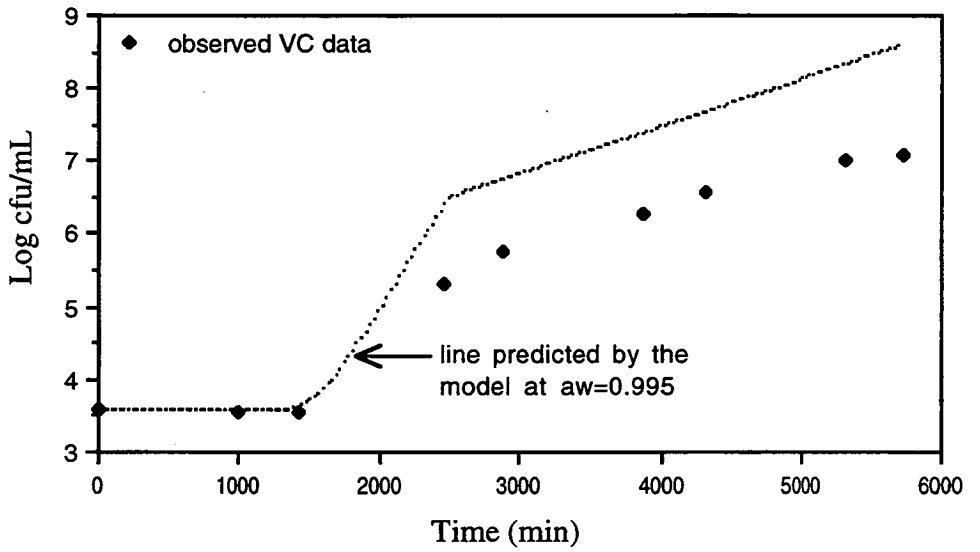
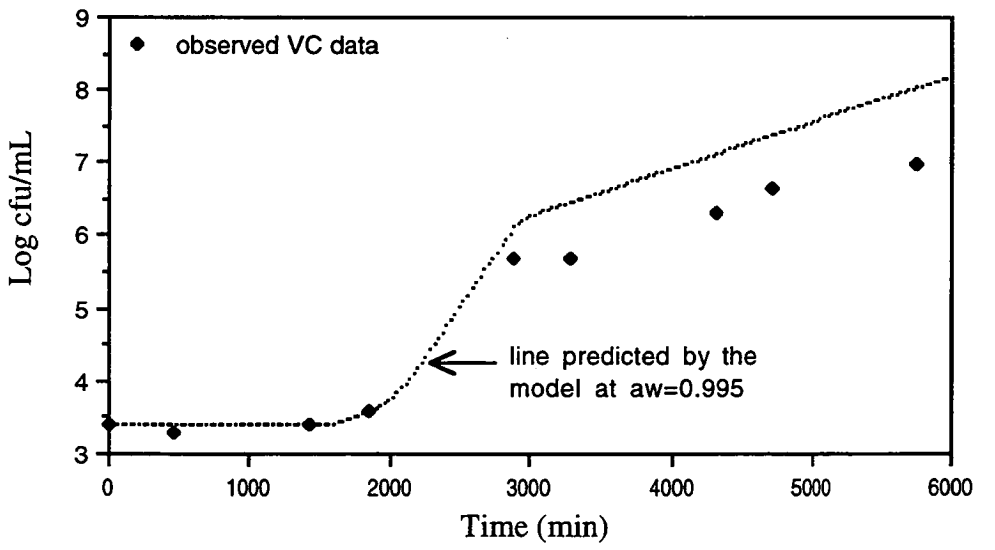


Figure 4.31 Observed viable count data and the growth curve predicted by the temperature/a_W model (at a_W=0.995) for the five trials (labelled a-e)

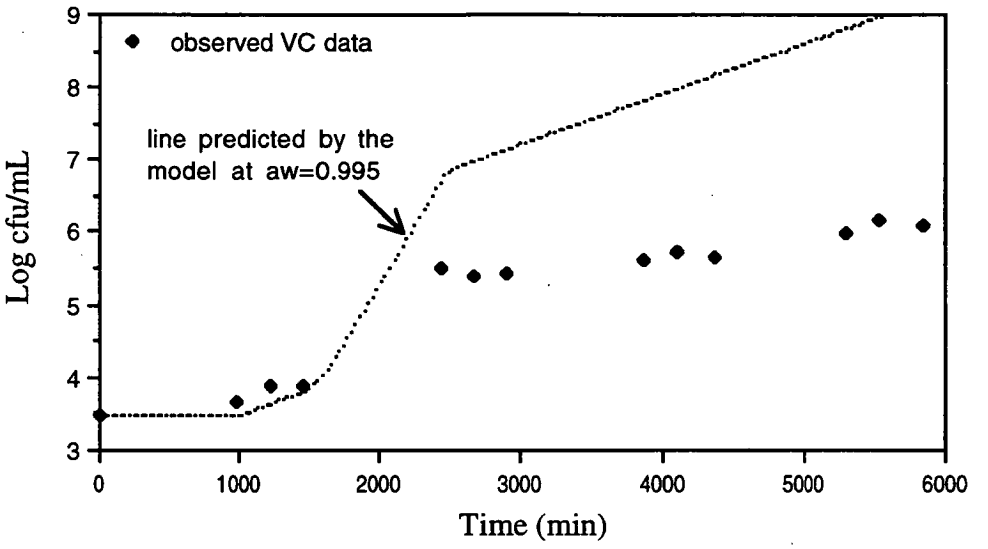
a) Trial #1



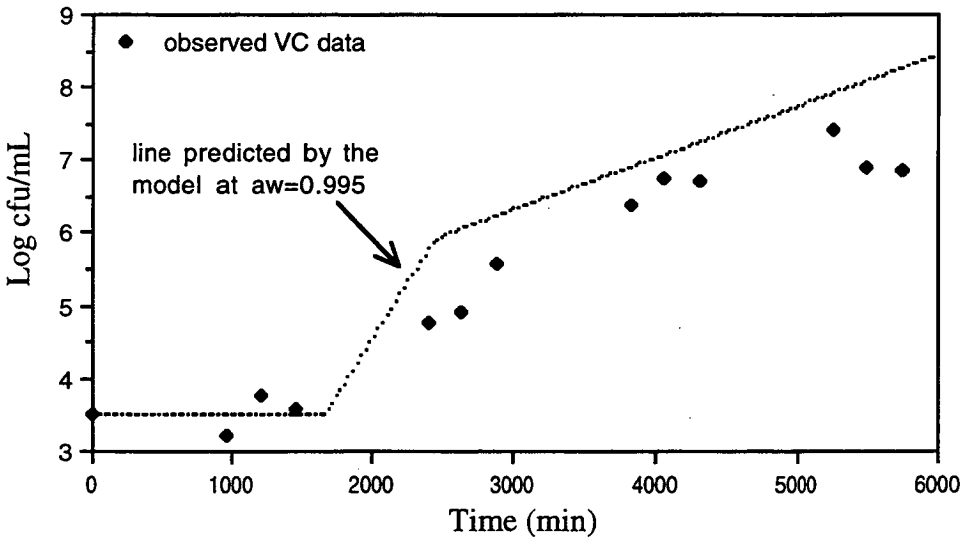
b) Trial #2



c) Trial#3



d) Trial #4



e) Trial #5

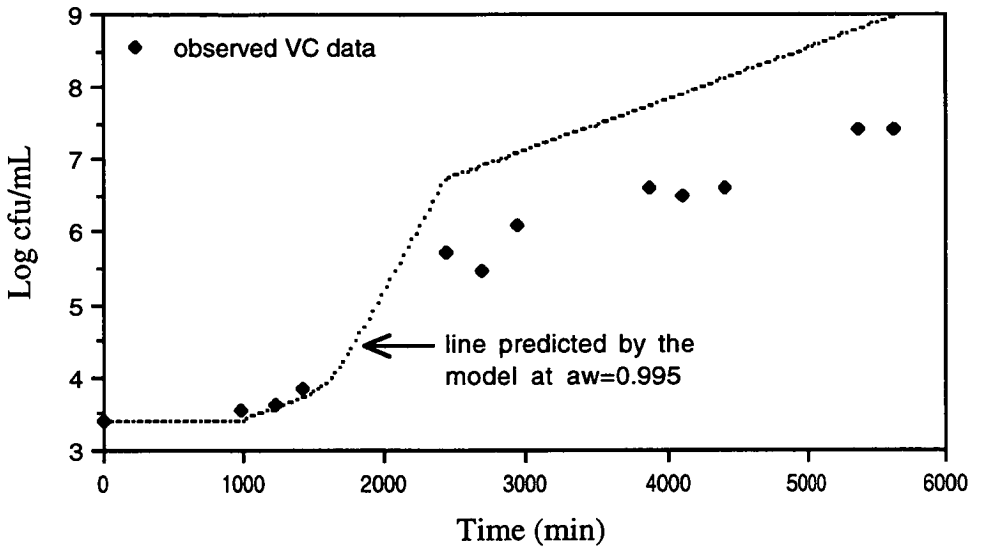


Table 4.17 Bias and accuracy factors for minced beef stored under fluctuating temperature conditions.

Trial Number	n	Bias	Accuracy
1	6	1.50	1.50
2	7	1.24	1.25
3	11	1.60	1.68
4	9	1.25	1.25
5	10	1.37	1.39
TOTAL DATA	43	1.39	1.42

4.6. INDUSTRY RESPONSE (QUESTIONNAIRE)

Eighteen (75%) questionnaires were returned. The degree of use '*Pseudomonas* Predictor' received is shown in Figure 4.32a. 83% of participants did not do validation trials. The main reasons for not completing trials are shown in Figure 4.32b. Despite the low rate of experimentation, 50% of 'clients' found applications for *Pseudomonas* Predictor within their company (Figure 4.32c). In some instances, the comment was made that although no direct applications had been found it was because they had not experimented with the package.

Figures 4.32d & e describe the user-friendliness of the manual and software respectively. Three respondents (17%) thought that they were not user-friendly. However, one stated that he 'found no computer manuals easy', the second that 'Excel version 5 is needed' and the third that, although he found it easy, for someone unfamiliar with microbiology (eg in a catering system or through the distribution chain) 'an integrated system where the user can input a particular food type rather than intial log count' was needed. Comments on how to improve the package included ' make it easier to show how to load in data manually', 'clarify the data file transfer area', 'need alternate versions to suit older and newer forms of lotus & excel'.

50% of respondents stated that they would buy *Pseudomonas* Predictor (Figure 4.32f). Of these, 6 (33%) thought the package was worth $200-400 while 5 (28%) placed its value at $400-600. This indicates that a reasonable price for *Pseudomonas* Predictor would be around the $400 price range. One respondent said that the price their organisation would be prepared to pay would 'depend on the revisions and the results of our validation trials'. Of those that stated they would not be interested in buying *Pseudomonas* Predictor, two said it wasn't relevant to their situation, two were involved in cheese manufacture and thought a package devoted to *Pseudomonas* was of limited use to them but would be interested if a 'Predictor' for a more relevant organism was available and the other one's comments indicate that he had not grasped the concept of predictive microbiology and *Pseudomonas* Predictor.

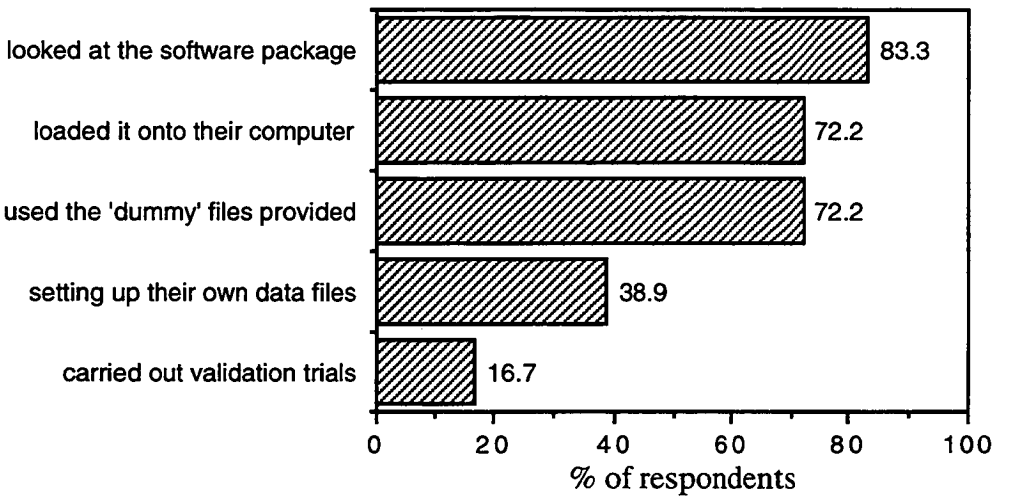
72% of respondents said they would use predictive models for other organisms if they were available. Some of the models requested were for Gram positives in extended shelf life products, thermophiles, yeasts & moulds, coliforms, *Listeria*, Enterobacteriaceae, lactobacilli, clostridia, *S. aureus*, *Salmonella* and *E. coli*. Some respondents were very unspecific and simply said 'other food spoilage and

food pathogens'. Few respondents mentioned environmental conditions of particular interest.

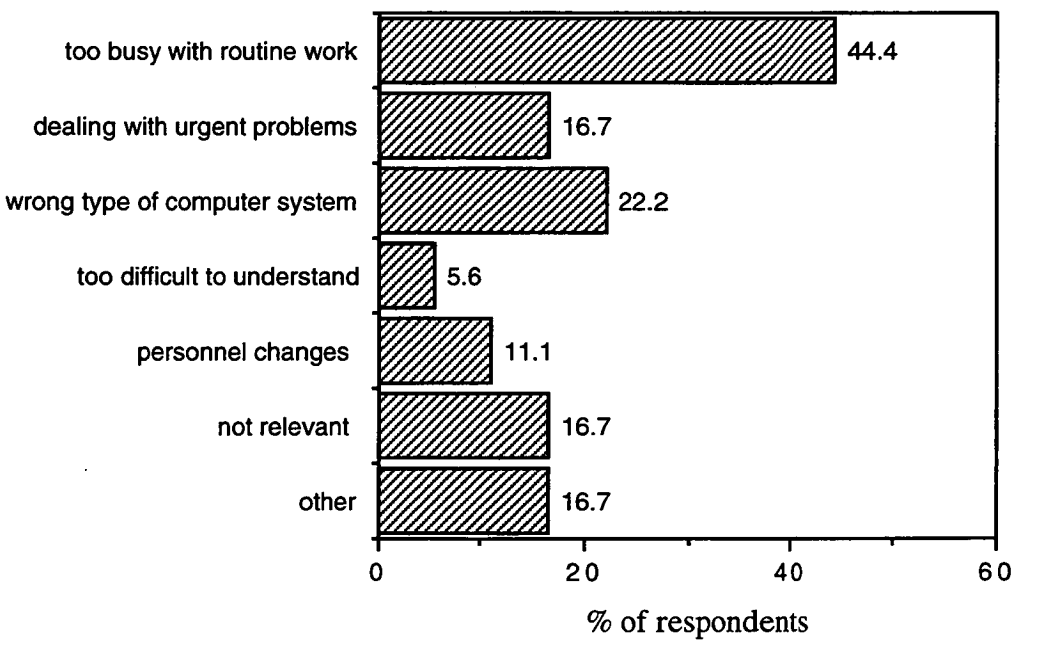
Although 67% thought '*Pseudomonas* Predictor' was a suitable name, the 28% that disagreed thought that the name needed to be 'glossed-up' and that it was too specific. One respondent thought that the name would mean 'nothing to a wider audience' and that 'a name that encompasses shelf life or safety would be more meaningful'. Only one respondent offered a suggestion, this being 'Food Microbe Predictor'.

Figure 4.32 Results of the Questionnaire about 'the *Pseudomonas* Predictor' package.

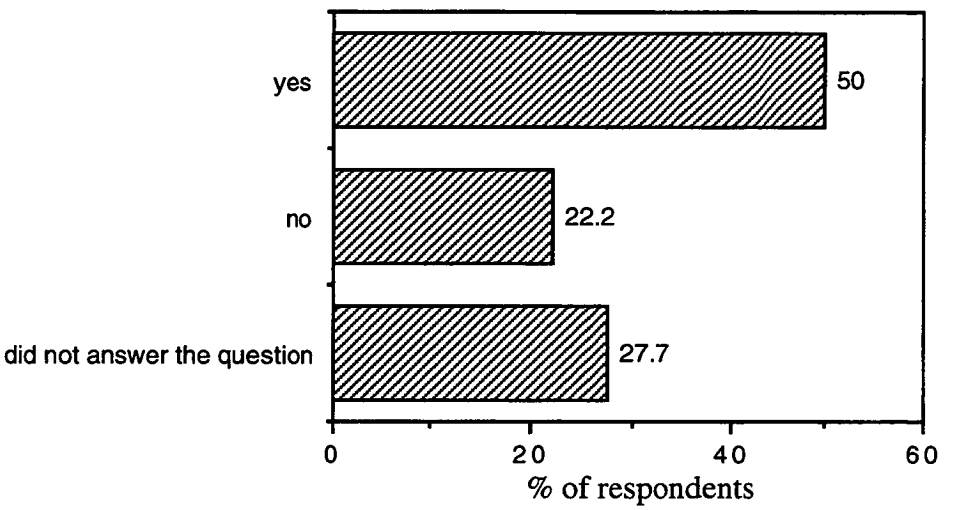
a.) How thoroughly did the participants use '*Pseudomonas* Predictor'?



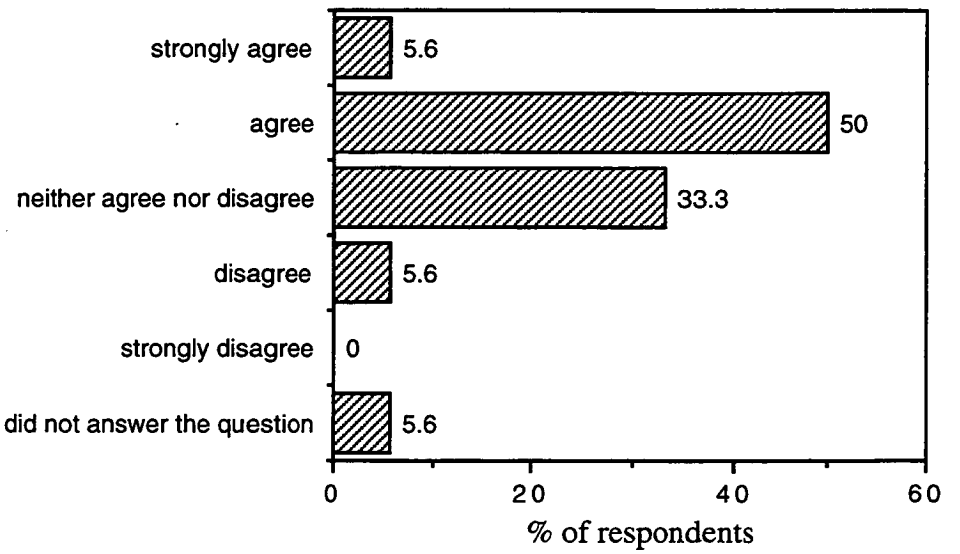
b.) What reasons were offered for not using '*Pseudomonas* Predictor'?



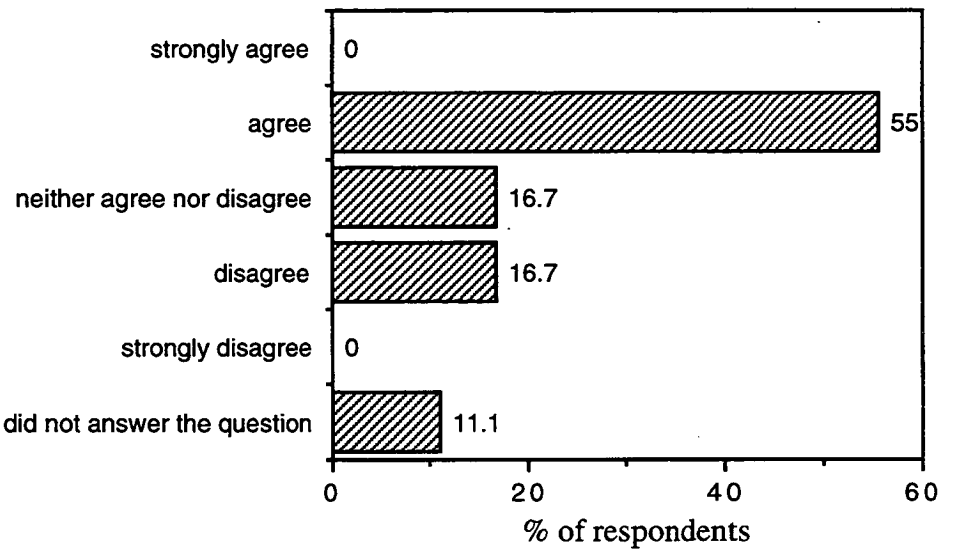
c.) Did the participants find any applications for '*Pseudomonas* Predictor'?



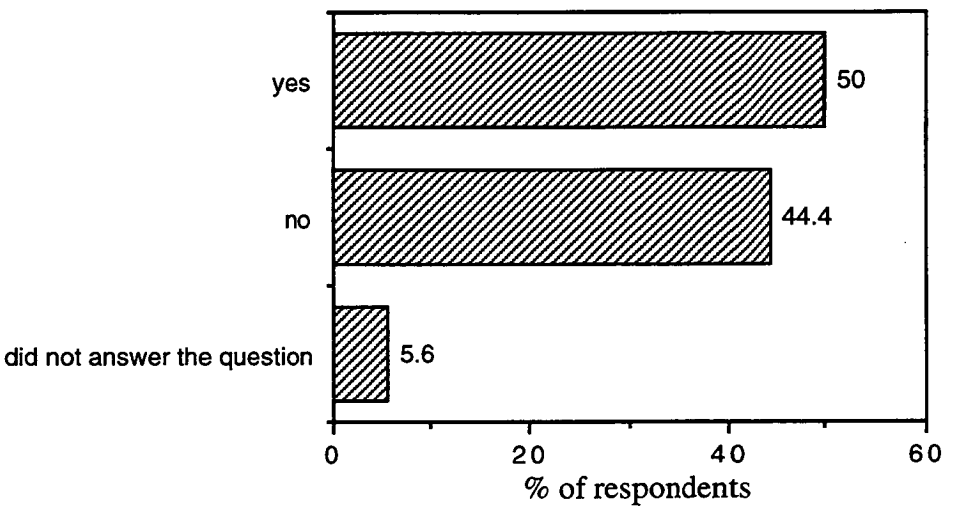
d.) Is the manual user-friendly?



e) Is the software user-friendly?



f) Would the participants buy the package?



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5. DISCUSSION

5.1. IDENTIFICATION OF PSEUDOMONADS

For modelling purposes it was necessary to isolate representative psychrotrophic pseudomonads from milk. It was therefore necessary to identify the strains as psychrotrophic pseudomonads but identification to species level was nonessential. The difficulty experienced in identifying isolates to species level using various growth characteristics is a common problem in pseudomonad research particularly amongst the fluorescent pseudomonads (Krieg & Holt, 1984; Lysenko, 1961; Palleroni, 1992). This problem was partially overcome by grouping pseudomonads into one of five "RNA similarity groups" and completing DNA-DNA hybridization studies on the pseudomonads (Palleroni, 1992). Under this system, *P. fluorescens*, *P. putida* and *P. fragi* all belong to rRNA Group 1 with *P. fragi* in a separate sub-group due to its nonfluorescence. Unfortunately no clear separation exists between *P. fluorescens* and *P. putida* and Palleroni (1992) suggests that they may represent different forms of a single species.

5.2. MODEL DEVELOPMENT

5.2.1. Temperature Models

5.2.1.1. Notional Minimum Temperature of Psychrotrophic Pseudomonads

An important characteristic of Bělehrádek type models is that the notional minimum temperature for growth (T_{min}) remains constant for a particular species. This characteristic is important as it is the basis of the relative rate concept (Eqn. 3.2) and provides the means whereby many models are incorporated into integrators. If models for psychrotrophic pseudomonads developed for milk and dairy products are to be considered universal, the T_{min} must remain the same regardless of the strain of pseudomonad and the food product the organism is isolated from. To test this assumption (that pseudomonads have the same T_{min} regardless of the food product that the strain was isolated from) a literature search was conducted to obtain T_{min} values from a variety of papers. In many instances growth rates were not discussed but other growth indicators were and data was obtained by reading graphs to get information of time versus, for example, shelf life (Foster *et al*, 1958), time to a flavour score <36 (Hankin *et al*, 1977) or time to increase two log cycles (Stannard *et al*, 1985). The data were graphed as square root plots to obtain approximate T_{min} values and divided into those from milk and flesh (beef, pork and chicken) sources. The summarised T_{min} data is shown in Table 5.1 with the complete information in Appendix 5. A t-test (2 sample assuming unequal variance) comparing the two groups of T_{min} data produced a t statistic of 0.1, well below the t_{crit} of 2.00, and a probability of 0.92 suggesting

that the psychrotrophic pseudomonads do have the same T_{min} regardless of what product a pseudomonad strain is isolated from. It is important to note that the T_{min} data was sourced from reports using different techniques, various conditions and a range of methods to calculate T_{min}. As a result, the 95% confidence interval (CI) was expected to be large. Contrary to these expectations, the 95% CI was quite small and the T_{min} ± 95%CI was 265.4K ± 0.7. As the data in Appendix 5 was compiled, it was noticed that as the size of the data set increased, the mean T_{min} values became more similar, the 95%CI became smaller and the statistical information improved (ie the t-statistic decreased and the probability increased of the hypothesis being correct). Thus as more data is added, the 'tightness' of the T_{min} estimate and therefore the 95%CI improves.

These results show that the T_{min} ± 95%CI for psychrotrophic *Pseudomonas* is 265.4K ± 0.7 regardless of the techniques used to determine growth rate and the source of the organism. This implies that only one model to describe temperature dependence is required for psychrotrophic pseudomonads regardless of the food product of concern although extra components for other environmental factors (e.g., pH and a_w) may need to be incorporated into the model for particular food products. For example, in liquid milk, temperature is the only factor of concern, water activity and pH both being optimal until after the product is considered spoiled (ie $>10^7$ cfu/mL), while in evaporated milk water activity is reduced and its effect must be incorporated into the modelling process.

Table 5.1 The summarised T_{min} data comparing pseudomonads from dairy origin with those from flesh sources.

Pseudomonad source	n	mean T_{min} (K)	SD	95% CI
dairy	35	265.43	2.69	0.89
flesh	29	265.36	3.03	1.10
all data	64	265.40	2.83	0.69

5.2.1.2. Model Generation

Modelling microorganisms in artificial broth systems using turbidmetric methods provides a rapid, inexpensive and reproducible means of determining bacterial growth rates. The reproducibility is demonstrated in Figure 4.2 in which datasets obtained on two separate occasions overlay almost exactly and provide a slightly better fit ($r^2 = 0.996$) than each dataset treated individually ($r^2 = 0.993$).

The ANOVA statistics for the 'b' values show that the various isolates are members of the same population with the F statistic being 0.40 (F_{crit} value of 5.19) and a probability of 0.80. This is confirmed by Figure 4.3 in which the mean 'b' values show little variation. Of interest are the almost identical average slopes for *P. putida* 1442 and the cocktail, even though one of the slowest strains (*P. fluorescens* 1412) was included in the cocktail. This suggests that the fastest strain (*P. putida* 1442) rapidly becomes dominant and further supports the decision to use the fastest strain on the basis of predicting growth rates using the worst case scenario.

In comparing the T_{min} values, F is greater than F_{crit} and P = 0.034 suggesting that not all isolates belong to the same population. However, with a probability between 0.05 and 0.01 the hypothesis is neither clearly proved or disproved. Figure 4.4 suggests that the inconsistant isolate may be *P. fragi* I6 which has a slightly higher mean T_{min} than the other isolates. If the *P. fragi* I6 data is removed and the ANOVA statistics reworked, F becomes 2.41 ($F_{crit} = 9.28$) and P = 0.244 signifying that the remaining isolates all belong to the same population. However, the pooled standard deviations of the mean T_{min} values of all isolates, including *P. fragi* I6, overlap suggesting that the inconclusive results produced by the ANOVA statistics may be due to the lack of replicates rather than being a true result. However, given that each square root plot contains approximately 30 growth rates, each one of which requires a minimum of 15 %T readings, resulting in some 450 (30 x 15) observations required for each TGI experiment and thus for each T_{min} and 'b' value, it is therefore impractical to obtain sufficient replicates of T_{min} and 'b' values for each strain for statistical validity. Given that the statistical studies on the 'b' value suggest only one pseduomonad population, and that psychrotrophic pseudomonads were found to have the same T_{min} regardless of the original source of the microorganism (refer to 5.2.1.1.), it is likely that *P. fragi* I6 belongs to the same population as the other isolates.

By determining the model over the entire biokinetic range, both the optimum and maximum temperatures can be obtained, these being 30.0°C and 40.7°C

respectively, for *P. putida* 1442. Above the optimum temperature the growth rates rapidly declined until no growth was detected at 40.7°C three days after inoculation. Modelling the entire biokinetic range shifts the value of T_{min}, for *P. putida* 1442, from -7.6°C (Figure 4.2) to -6.32°C (Figure 4.2). This variation is within the range observed in Figure 4.4.

5.2.2. Water Activity Models

5.2.2.1. Calculation of Water Activity

Water activities determined using the Aqualab CX2 agreed very well with those calculated theoretically. The slope of 0.97 and an $r^2 = 0.999$, shown in Figure 4.6, suggest that there is some systematic deviation of the mathematically derived a_w from those observed by the Aqualab. Over the range tested (0.94 - 0.999) the effect of this error was less than the accuracy of the Aqualab (± 0.003 a_w units). It was therefore unnecessary to determine the water activity of each broth and the a_w of the broths were derived mathematically. Stamp *et al* (1984) found similar results when various methods of determining water activity were tested. It was found that for each method, the slope was close to but not exactly one and that the intercept was, therefore, not at zero. This error was attributed to each individual instrument and that any device of the same model would have its own regression equation.

5.2.2.2. Model Generation

For all strains the observed generation times were shorter than those described by the model for that strain at the lowest water activity (e.g., Figure 4.7) with the effect that growth is observed below the predicted $a_{w\ min}$ value. This is possibly due to increasing variance as the trend is similar to that observed as temperatures approach the growth/no growth boundary in the temperature experiments. At these extremes there is a possibility for both over- and under- predictions however often only the underpredictions are detected as the experiment may be abandoned before growth in the very slow growing cultures is detected.

The ANOVA statistics, with probabilities well above the 0.05 level, suggest that the isolates belong to the one population. This is confirmed by the comparison of the $a_{w\ min}$ values in Figure 4.9. However, in Figure 4.8 *P. putida* 1261 appears to have a lower 'b' value than the other isolates. As the probability of the isolates belonging to one population is 0.373 and the pooled standard deviations overlap slightly it is likely that further work would show that *P. putida* 1261 belongs to the same population.

5.2.3. Combined Temperature / Water Activity Models

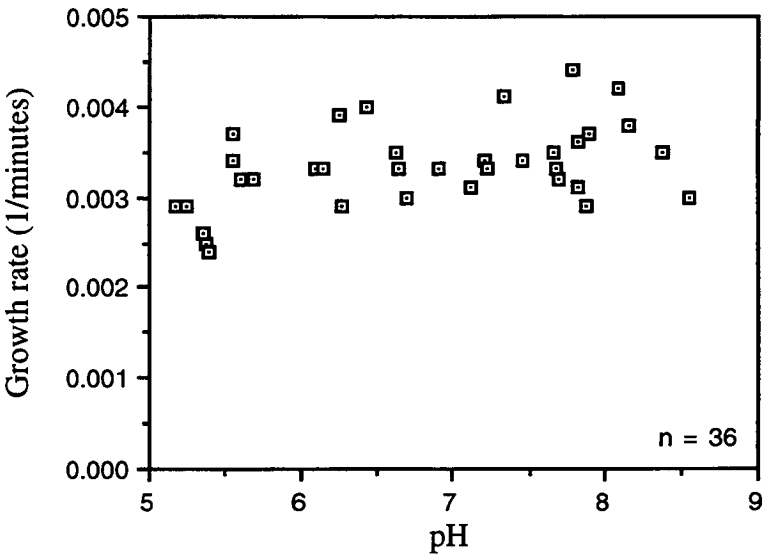
Figure 4.10 shows that as the water activity decreases the observed data falls further away from the model predictions. This is most clearly seen in the standardised residuals of the a_w=0.960 data, in Figure 4.11, where the trend is towards positive values, rather than a balanced mix of positive and negative values. The other water activities also show odd trends. For example, both strains at a_w=0.969 show a positive trend at the higher temperatures. Importantly the data for a_w=0.996 shows little scatter and has a balance between positive and negative residuals suggesting that there may be a problem with the model at environmental extremes rather than in the experimentation. This is further borne out in the data from Table 4.6 where the bias and accuracy values increase as the a_w decreases. It may be that the amount of error observed is not important for the modelling process as the bias and accuracy of the combined data is around 1.06 and 1.18, respectively, for each strain and is therefore within the 20% error margin. Ross (1995) maintains that it is also necessary to consider the pattern of the standardised residuals when considering the bias and accuracy factors as a systematic deviation may result in low bias and accuracy factors due to under- and over- predictions cancelling one another out. The standardised residuals in Figure 4.11 suggest that there may be some systematic deviation at higher temperatures and that the deviation is greater as water activity decreases. Further experimentation is required to achieve a definitive conclusion as to whether the deviations are insignificant or if there is a water activity/temperature related trend away from predicted values.

5.2.4. Effect of pH on the Growth of Pseudomonads

Figure 4.12 shows that there is a trend such that as pH declines from pH 8.0 to 5.4 growth rate decreases from ≈0.010 to ≈0.007. The observed trend may be a result of insufficient data as Kamperman (1994) showed *P. fluorescens* strain 3 to be unaffected by pH changes in the range pH 5.4 to 8.6 (Figure 5.1). Growth rates were significantly slower at pH 5.5 and below. Kamperman (1994) explains that the difference in growth rates at pH 5.4 to 8.6, ranging from 0.003 to 0.004 at 10.8°C, is within the variances expected at these rates. This suggests that pH does not need to be considered in the modelling of psychrotrophic pseudomonads as most products on which pseudomonads are the main organism of concern have a pH between 6 and 8. If pH is of concern in a particular product the model would err on the side of safety, as products with a lower pH will have slower growth rates. It may be necessary, at a future time, to incorporate a pH term into '*Pseudomonas* Predictor' in order for the model to be more widely applicable.

The observation that the growth rates of pseudomonads decline markedly below pH 5.4 contradict the literature in which *Pseudomonas* is considered to be one of the main spoilage organisms of cottage cheese despite the pH of cottage cheese ranging from <5 to 5.3 (Kosikowski, 1970). Kamperman (1994) calculated that the concentration of undissociated acid, at pH 6.4 and 0.2M lactate, to be 0.58mM. Houtsma *et al* (1993) found the minimum inhibitory concentration of sodium lactate for the growth of pseudomonads to range from 714mM to 982mM. Given that the pK_a of lactic acid is 3.9 (Ross, 1981), the equivalent undissociated acid concentration, for the Houtsma *et al* (1993) experiments, ranged from 2.1 to 2.8mM, which is far higher than 0.58mM used in these experiments. C. Aalberts (*pers. comm.*) suggested that the concentration of lactate may affect the pH_{min} observed as the pH_{min} of *Staphylococus aureus* was 4.8 when using 0.2M lactate, while at low lactate concentrations (0 - 0.5M), the pH_{min} dropped to approximately 4.0. Thus, while the concentration of lactate, at 0.2M may not be inhibitory to the growth of pseudomonads, it may affect the pH_{min} at which growth is observed.

Figure 5.1 Effect of initial pH on the growth rate of *P. fluorescens* strain 3 at 10°C (*adapted from* Kamperman, 1994).



5.3. MODEL CALIBRATION

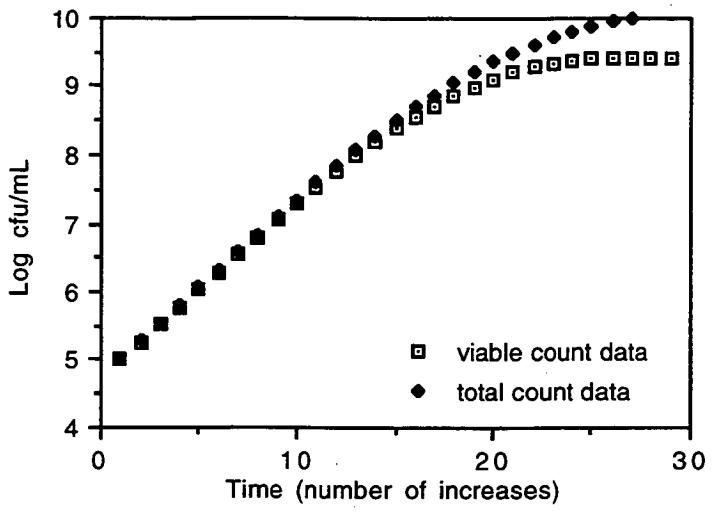
Although estimates of generation time from VC are faster than those determined by %T, the difference is constant (r^2 = 0.026 in Fig. 4.13) over the temperature range studied (4 - 26°C). Therefore only a simple calibration factor (1.50 from Table 4.7) is necessary to adjust generation times calculated by %T to those calculated by VC methods ie,

$$GT_{VC} = GT_{\%T} / 1.50 \qquad (5.1)$$

A possible reason for the difference between GT_{VC} and $GT_{\%T}$ could be that non-viable cells are contributing to the the increase in turbidity of the %T data, thereby showing slower generation times than are actually occurring. Viable count methods do not detect the proportion of non-viable cells in a culture. This hypothesis was tested with the use of a computer simulation. Figure 5.2 was plotted using several assumptions, these being that the proportion of non-viable cells in an exponentially growing population was 10% and that once viable numbers had reached 10^7 cfu/mL the proportion of non-viable cells increased at a rate of 2.5% per doubling until a maximum rate of 50% was reached, thereby simulating the approach to maximum population density. In this instance the generation time between the viable counts and total counts (ie viable plus non-viable counts) are almost identical suggesting that the effect of the non-viable cells are minimal. By varying the assumptions it was possible to change the curves produced but in no instance did the viable counts increase at a faster rate than the total counts thereby discounting the original hypothesis. This hypothesis could also be checked experimentally using flow cytometry methods eg detect the total counts using normal methods and the viable counts using vital stains with the addition of a cell sorter.

Another possible explanation for the observed difference between GT_{VC} and $GT_{\%T}$ could be that the spectrophotmeter will only read within the range 10^7 cfu/mL (from Figure 4.14) to $\approx 5 \times 10^8$ cfu/mL (Ross, 1993) where the culture is already close to the maximum population density and growth rates may already be declining. Many workers (Jason, 1983; Mackey & Kerridge, 1988; Buchanan & Phillips, 1990; Neumeyer, 1992) have found no evidence of inoculum size on generation times, however, the range of initial inoculum sizes tested by these authors ranged from $\approx 10^{-2}$ cfu/mL to $\approx 10^5$ cfu/mL, with one exception at 10^7 cfu/mL. Ross (1993), however, investigated the effect of initial inoculum levels on the generation times for both %T and VC data for *Listeria monocytogenes*

Figure 5.2 A computer simulation of growth of viable and total counts over time assuming a 10% proportion of non-viable cells and that above 10^7 cfu/mL the proportion of non-viable cells increases at a rate of 2.5% per doubling until a maximum rate of 50% is reached.



Scott A and *Staphylococcus aureus* 3b at initial levels ranging form 2.5 to 8.5 log cfu/mL. Although the ratio ($GT_{\%T}/GT_{VC}$) was close to one for the *S.aureus* data at high initial population densities (log7.4 cfu/mL), no such relationship was found for *L. monocytogenes* at high initial population densities (log8.5 cfu/mL).

The overall ratio of 1.44 ± 0.24, from Table 5.2, suggests that it may be possible that the ratio may be constant for all bacterial species, although, more data is needed for some of the species in particular those in which the results are based on only 4 - 6 growth curves. A more likely scenario is that each species has its own individual ratio value and that this difference may be due, in part, to the size of the bacterial cell. This may explain the ratio of 1.80 reported for *Photobacterium phosphoreum* as Dalgaard (*pers. comm.*) had noted that it was an extremely large bacterium. Furthermore Maaløe & Kjeldgaard (1966) showed that the cell mass is related to the growth rates of bacterial cells such that fast-growing cells are large while slow-growing or resting cells are small. This phenomenon is explained in some detail in Neidhardt *et al* (1990). One example provided by Neidhardt *et al* (1990) is that the average *E. coli* cell grown at 2.5 doublings per

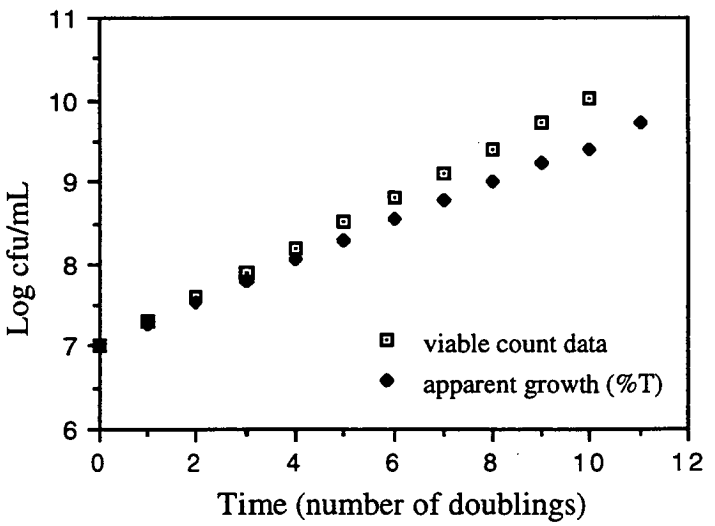
hour has almost six times greater mass than the average *E. coli* cell grown at 0.6 doublings per hour. This difference in cell mass is dependent on the growth rate of the bacteria and will occur regardless of what causes the decline in growth rate, ie either temperature (as in the *E. coli* example) or the approach to MPD, will cause a decline in growth rates and therefore a decrease in the cell mass. As the spectrophotometer only monitors growth above 10^7 cfu/mL it may be that the difference in cell mass, and therefore turbidity, is contributing to the difference in generation times determined using %T and VC methods. This hypothesis was tested using a computer simulation in which it was assumed that the cell mass was four times larger in exponential phase than at MPD and that the proportion of cells with a smaller mass increased 10% per doubling. Figure 5.3 shows that following these assumptions the growth rate of the bacterial population does appear slower than that compared to viable counts. If the data is analysed using the Gompertz function, the viable count population has a generation time of 0.8889 (time in arbitrary units) while the generation time calculated from the cell mass assumptions is 1.1404 (time in arbitrary units) resulting in a $GT_{\%T}/GT_{VC}$ ratio of 1.28. While this does not account for all of the difference between $GT_{\%T}$ and GT_{VC} it does appear to contribute and the difference between $GT_{\%T}$ and GT_{VC} may be due to a combination of both the cell mass and the limits of the spectrophotometer. Experimental analysis is required to further test the cell mass hypothesis.

Table 5.2 Ratio ($GT_{\%T}/GT_{VC}$) for various bacterial species.

Species	Average Ratio ($GT_{\%T}/GT_{VC}$)	SD	Number of Growth Curves
Listeria monocytogenes Scott A[1]	1.23	0.16	17
Staphylococcus aureus 3b[1]	1.28	0.17	6
Photobacterium phosphoreum[2]	1.80	0.71	6
Shewanella putrefaciens[2]	1.28	0.07	4
Vibrio paramhaemolyticus strain 38.349[3]	1.41	0.13	6
Pseudomonas spp[4]	1.58	0.21	16
Pseudomonas spp[5]	1.50	0.15	12
Total	1.44	0.24	67

1 Ross (1993)
2 Dalgaard *et al* (1994)
3 Miles (1994)
4 Kamperman (1994)
5 from Table 4.7

Figure 5.3 A computer simulation of viable growth and apparent growth rates based on the assumption that cell mass decreases as the growth rate slows.



5.4. MODEL VALIDATION (LABORATORY STUDIES)

Observed and predicted results can be compared by a number of methods. Plotting the residuals is one method utilised as the plots are useful in diagnosing any non-linearity or non-constant error variance in the models, however, they do not provide a means whereby models can be compared in a numerical fashion. For this, bias and accuracy factors were used. Of particular interest, was comparing the model (Equation 4.3) to literature pseudomonad growth rates. However, before the model was compared to the literature, it was first compared to the data used to generate the model itself. By comparing the generation time data of a strain with the generation times predicted by the model of that strain, an understanding of a 'perfect score' is developed. In this situation bias must be 1.0, while accuracy will reflect the extent of experimental error. This is shown in the results of Table 5.3 (bias 1.0; accuracy ≈ 1.05). Where the strains (cocktail, *P. fragi* NCIMB 8542, *P. fragi* I6 and *P. fluorescens* 1412) are compared to the *P. putida* 1442 model (Table 5.4) both the bias and accuracy values increase with *P. fragi* NCIMB 8542, *P. fragi* I6 and *P. fluorescens* 1412 having bias and accuracy factors of 1.15 - 1.20. The cocktail has lower bias and accuracy factors of 1.06 and 1.10, respectively, reflecting the dominating presence of *P. putida* 1442 in the cocktail culture.

In Table 5.5 the *P. putida* 1442 model is compared with pseudomonad generation times calculated from literature data. In many cases insufficient information regarding the growth conditions was provided, for example, there was often no indication of the degree of aeration, how well temperature was controlled or the number of samples taken per growth curve, thus making valid comparisons between observed and predicted generation times difficult. An overall bias of 1.31 and a precision of 1.36 was higher than anticipated and probably indicates the problems inherent in some of the literature data. For example, the data from Shelley *et al* (1986) has bias and accuracy factors of ≈1.70 suggesting that the model for *P. putida* 1442 is not appropriate in that situation. The data, however, was quite poor; there were only ≈7 readings taken per growth curve, growth conditions were probably not optimal as there is no mention of any oxygenation and the data was obtained from a graph that was, initially, very small. McClure *et al* (1994) also noted that observed growth rates were faster than literature values and attributed the difference to limiting factors in the literature experiments. Ross (1995) suggested that it may be more appropriate to use data that has been derived in controlled conditions rather than using literature data so that the models performance is not unfairly prejudiced by comparisons to unrepresentative or poor

Table 5.3 Bias and accuracy values obtained by comparing the generation time data generated in nutrient broth of a pseudomonad strain to the generation times predicted by the model of that strain.

Strain	Bias	Accuracy	n
P.putida 1442	1.00	1.05	56
Cocktail (5 strains)	1.00	1.06	58
P.fragi NCIMB 8542	1.00	1.03	56
P.fragi I6	1.00	1.05	90
P.fluorescens 1412	1.01	1.06	30

Table 5.4 Bias and accuracy values of pseudomonad strains tested against the model for *P. putida* 1442 in broth.

Strain	Bias	Accuracy	n
Cocktail (5 strains)	1.06	1.10	58
P. fragi NCIMB 8542	1.15	1.16	56
P. fragi I6	1.19	1.20	90
P. fluorescens 1412	1.15	1.17	30

Table 5.5 Bias and accuracy factors of literature data tested against the model for *P. putida* 1442 developed in broth.

Author	Bias	Accuracy	n
Growth in milk or using milk derived pseudomonads			
Griffiths & Phillips, 1988b	1.08	1.11	3
Langeveld & Cuperus, 1980	1.09	1.09	10
Maxcy & Liewen, 1989	1.22	1.22	6
Robinson, 1981	1.55	1.55	4
Shelley *et al,* 1986			
- AS7c1	1.71	1.71	6
- AS11a1	1.68	1.68	6
- AS31a1	1.82	1.82	6
- AS24b1	1.68	1.69	6
COMBINED	1.44	1.44	47
Growth in meat or using meat derived pseudomonads			
Ayres, 1960	1.79	1.79	5
Delaquis & McCurdy (1990)			
- *P. fluorescens* CC-841406E	1.27	1.28	2
- *P. fragi* D5	1.42	1.42	2
Gill & Newton, 1977			
- fluorescent pseudomonads	1.09	1.14	4
- non-fluorescent pseudomonads	1.14	1.15	4
Pooni & Mead, 1984			
- pigmented	1.07	1.08	6
- non-pigmented	1.02	1.08	6
Scott, 1937			
- No.7	1.16	1.39	8
- No. 483	1.18	1.33	8
- No. 5	1.25	1.28	6
- No. 451	1.40	1.40	8
COMBINED	1.23	1.30	59
MILK & MEATS COMBINED	1.31	1.36	106

quality data. Importantly, in all cases, the model is 'fail safe' suggesting that the strain used to develop the model (*P. putida* 1442) is faster than others described in the literature.

5.4.1. Milk

The trend for pH to start decreasing at approximately 10^9 cfu/mL (Figure 4.15) provides an indication of when MPD is approaching and thus when to cease milk sampling during growth experiments. A similar trend of the pH response during milk spoilage occurred at all temperatures tested, although Figure 4.16 shows that a slight temperature related trend may exist ($r^2 = 0.781$). However given that viable count methods have a variability of ±0.5 log cycles (Jarvis, 1989) the trend is unlikely to be significant.

Figure 4.17a indicates that the model accurately predicts generation times observed in pseudomonads in milk with optimal aeration but that at minimal aeration (Figure 4.17b) the generation times appear to be slightly longer than those predicted. The situation is more clearly determined in Figure 4.19 in which the standardised residuals for milks under optimal aeration show a balanced scatter of positive and negative residuals while those for minimal aeration are almost all positive. Despite this positive trend the residuals for pseudomonads in non-agitated milk are not outside the range of those for pseudomonads in the aerated milk products.

Figure 4.18 shows a pattern of non-constant variance with temperature, where the variation of the residuals increases as the temperature decreases. Similar results were observed in data used by Ratkowsky (1992) who proposed that the variance of the generation time is proportional to the squares or cubes of their means and that linear regression models should therefore be described using "gamma" or "inverse Gaussian" error. Incorporating these error terms into the concept of predictive microbiology leads to the idea that the probability of growth at the described environmental conditions should be calculated rather than necessarily predicting the exact generation time so that the user decides the level of risk he is willing to assume in his product. This would be especially important in situations of harsh environmental conditions such as low temperature where the variance becomes very large. For example, Ratkowsky (1992) describes a situation in which, at 10°C, if a risk of 10^{-3} or less was acceptable, a generation time of 2.55 hours (for *E. coli* on meat) should be used for predictive modelling. If this level of risk was not stringent enough and a risk of 10^{-6} was applied, the generation time used for modelling becomes 1.55 hours. From this example it is clear that the

lower the acceptable risk, the shorter the generation time and thus the shorter the 'safe' storage time, or shelf life, of the product becomes.

The growth of pseudomonads in milk, for which the samples of milk were inoculated with *P. putida* 1442, resulted in bias and accuracy factors of approximately 0.98 and 1.08 (Table 4.10). The uninoculated raw milk, with a bias of 1.03 and an accuracy of 1.13, brought the combined value of pseudomonads in milk under optimal laboratory conditions to a bias of 0.99 and an accuracy of 1.08. These results indicate that the model accurately predicts the growth of pseudomonads in the laboratory and is well within the 20% error margin that Walker & Jones (1994) consider to be an achievable aim for predictive microbiology. The results for bias and accuracy also show that the model is not underpredicting the extent of growth and thus is 'fail safe'.

P. putida 1442 in modified milk with minimal aeration grew slower than when grown with optimal aeration resulting in the bias and accuracy values of 1.17 suggesting that it may therefore be necessary to incorporate degree of aeration into the model. Similar results were found by Lambert *et al* (1991) who found pseudomonads on meat at 3°C to grow 20-30% slower in 20%CO_2 and 80%air compared to the same strain grown in 100% air. Despite the slower growth rates, the bias and accuracy values are roughly equivalent to those of other strains when compared to *P. putida* 1442 in Table 5.4. It is therefore necessary to determine whether the degree of aeration will effect the growth of pseudomonads in industrial situations, that is, does the slowing of the growth rate simply increase the 'fail safe' margin or does the effect of oxygen need to be incorporated into the modelling process? In the dairy industry it is unlikely that the degree of aeration will be an important factor as, in most situations, the milk, or its derivative, is agitated in order to maintain homogeneity.

5.4.2. Evaporated Milk

From Figure 4.20 it can be seen that observed generation times derived from PCA counts deviate from those predicted by the model above approximately 19°C, while the generation times calculated from the PSA data do not. Figure 4.21a suggests that, as PSA counts are higher than PCA counts, the difference is not due to non-psychrotrophic contaminants. The difference between PSA and PCA counts becomes less pronounced as the temperature decreases until at 2.8°C PSA and PCA counts are the same (Figure 4.21b). It was hypothesized that the differences observed at high temperatures may be due to the differences in water activity of the culture media itself. PCA has a a_w of 0.997 while the a_w of PSA is

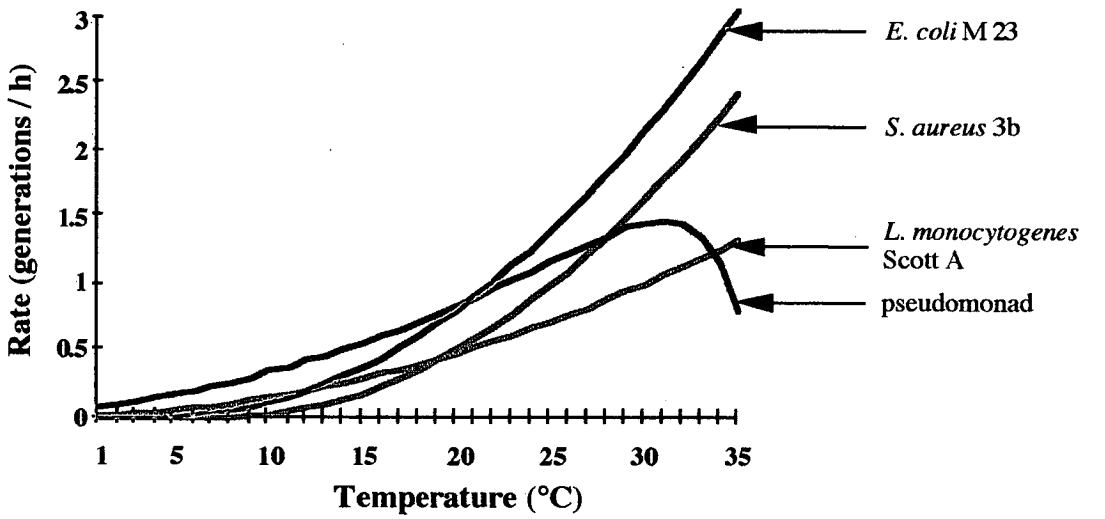
0.988 (the same as evaporated milk). While the a_w difference appears slight, the generation time, at 25°C, is 1.25 times longer at an a_w of 0.988 (GT = 55 minutes) than at an a_w of 0.997 (GT = 44 minutes). Thus at high temperatures, aliquots taken from evaporated milk and plated onto PCA may experience a lag phase due to the change in a_w conditions. *P. putida* 1442 plated onto PCA are large mucoid colonies and so the bacteria that enter exponential phase faster may overgrow the bacteria with longer lag phases resulting in falsely low viable counts. The reverse (PSA counts being incorrect) was not observed in milk possibly because colonies on PSA do not exhibit the mucoid tendency shown on the PCA plates and thus the colonies remained distinct (and countable).

Given these results, PCA generated data above 19°C were not used for the purpose of comparing observations to predicted generation times, ie only PCA results <19°C and PSA results are used. Table 4.11 shows that the bias and accuracy factors for both the PCA<19°C and PSA data are within a 10% error margin. If all the data is combined the bias and accuracy become 1.00 and 1.08, respectively, indicating that the observed and predicted values agree. The standardised residuals are between 0.01 and -0.01 and show no trends (Figure 4.22a) confirming the goodness-of-fit of the model and indicating that no systematic deviation of the model exists for pseudomonads in evaporated milk.

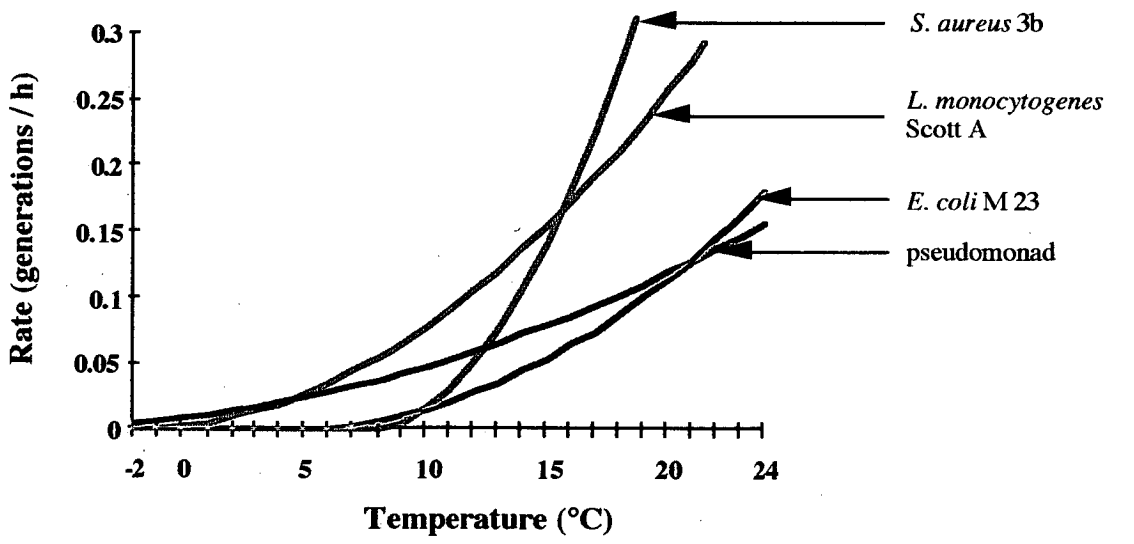
From an industry perspective it may be necessary to use both probability and kinetic models - probability models for pathogens where any growth is of concern and kinetic models for spoilage organisms. Once environmental conditions become harsher to the extent that variances of growth rates of the dominant microorganism start increasing it is unlikely that the original spoilage microorganism will remain the organism of concern in a particular product. For example, although pseudomonads can grow at an a_w of 0.96, they are unlikely to remain the dominant biota as other organisms will be able to grow much faster under the same conditions. This is shown in Figure 5.4 in which models for *Pseudomonas*, *Listeria monocytogenes*, *Staphylococcus aureus* 3b and *Escherichia coli* have been plotted together at water activities of 0.99 and 0.96 (Ross, unpublished). While pseudomonads are dominant at an a_w of 0.99 until nearly 20°C, *L. monocytogenes* and *S. aureus* rapidly outgrow the pseudomonds at the lower water activity.

Figure 5.4 Comparison of growth rates at water activities of 0.99 (a) and 0.96 (b) using NaCl as a humectant (Ross, *unpublished*).

a



b



5.4.3. Cream

Although pseudomonads in cream were not initially the dominant microorganisms, Figure 4.23 shows that they quickly grew to overtake the other microbiota. This did not occur at 15°C where pseudomonads are displaced as the dominant organisms by other organisms to the extent that pseudomonads were not detected at the dilutions plated.

The measured water activity of cream, at 0.995, differs greatly from the literature. Chirife & Ferro Fontan (1982) give the a_w of cream as 0.979 using the cryoscopic temperature method as described in Rha (1975). The Aqualab CX2 uses a primary technique (the dew point) for determining a_w while many other available methods do not. As such, many are unable to determine a_w values above 0.98. This is the region where an accurate reading is necessary for modelling pseudomonads, as is the ability to determine a_w to 3 significant figures. Not doing so can change the predicted a_w value and, as a result, the predicted generation time. For example, the predicted generation times for pseudomonads at 6°C and a_w of 0.984 is 347 minutes or 310 minutes using 2 and 3 significant figures respectively.

The square root plot of the growth of pseudomonads in cream (Figure 4.24) at the three temperatures (2, 6 and 11°C) shows that the observations fit the line predicted by the model reasonably well. The standardised residuals for brand 2 shows no trend, although there may be a pattern of systematic deviation for brand 1. It is less likely that deviations will occur in cream inoculated with *P. putida* 1442 (the strain with which the model was developed) than when the natural biota is monitored. The systematic deviation observed in brand 1, the cream inoculated with *P. putida* 1442, is, therefore, more likely to be due to insufficient data points rather than being a true trend. The bias and accuracy factors in Table 4.12 show that the model predicts the growth of pseudomonads in cream to within 20% error. Not surprisingly the cream inoculated with *P. putida* 1442 had better bias and accuracy estimates than the natural biota of the cream. This was also observed in the unpasteurised whole milk (Table 5.6). It is important to note that the model did not predict in a 'fail dangerous' fashion but rather maintained the required 'fail safe' feature.

5.4.4. Comparison of Observed and Predicted Data

Evidence that as more and more data is obtained the bias and accuracy factors become closer and closer to 1.0 is shown, in particular, by the cream (n=3) and low fat milk (n=34) validation studies where bias and accuracy factors were much closer to 1.0 in the latter instance. Similarly if all the laboratory validation studies are collated into one table (Table 5.6), the bias and accuracy become 1.00 and 1.09, respectively, indicating that the model accurately predicts the growth of pseudomonads in the products tested.

Table 5.6 Bias and accuracy factors for all laboratory validation studies.

Food Type	n	Bias	Accuracy
Low Fat Milk	34	0.98	1.07
Whole Milk			
- pasteurised	21	0.98	1.08
- unpasteurised*	13	1.03	1.13
Evaporated Milk	11	1.01	1.08
Cream			
- inoculated	3	1.06	1.15
- uninoculated*	3	1.19	1.19
TOTAL DATA	85	1.00	1.09

* = natural biota monitored

5.5. MODEL VALIDATION (INDUSTRY STUDIES)

5.5.1. 'Client' 1: Raw Milk

The milk cooling vats were continually agitated to prevent the formation of a cream surface. This removed any possibility that the degree of aeration may present a problem and cause a deviation from predicted results. Additionally any effect due to lack of oxygen would decrease the growth rate observed and not simply increase the lag phase. One explanation, for the extended lag phase may be that the lactoperoxidase system is operating. Discussions with the microbiologists at the processing plant suggest that the region contains large amounts of capeweed

which may be resulting in high natural concentrations of thiocyanate. As discussed in 1.1.1.3.2. the lactoperoxidase system affects the length of the lag phase but once growth starts, the growth rates are as expected. Further work is required to study this hypothesis.

Although the results from Table 4.14. are by no means conclusive, (particularly as Farm 1 Trial 2 does not contain statistically valid data) the bias and accuracy factors of 1.12 suggest that the *Pseudomonas* model will accurately predict the extent of growth of pseudomonads provided the duration of lag phase is known for the raw milk samples.

To obtain more complete validation data, a situation where cooling will not be improved upon once experimentation begins (even though the farmers were asked not to change any current procedure), and where the lactoperoxidase systems will no longer be active, was sought. Two locations were possible. The first was during milk storage in the silos at the processing plant, while the second option was monitoring tanker trips of unpasteurised milks that take longer than 5 hours. The first option is simpler, as microbiological testing can be carried out on site immediately by the microbiologist. Unfortunately due to urgent and routine work and a change in personnel no further validation trials were carried out during the '*Pseudomonas* Predictor' trial period.

5.5.2. 'Client' 2: Reconstituted Whole Milk and UHT Milk

The main problem with the data from 'client' 2 was that only 6 - 8 samples were taken per growth curve which is under the 10 - 15 readings recommended by Gibson *et al* (1988), Labuza & Fu (1993) and McMeekin & Ross (1993). This was a problem in almost all the data collected from the various 'clients'. Bias and accuracy information on the data from 'client' 2 is shown in Table 5.7 and is discussed more fully in 5.5.5.

5.5.3. 'Client' 3: Cream

The data from 'client' 3 on cream is quite poor and no conclusions can be considered statistically valid. Problems occurred in lack of consistency of how the temperature was taken (ie depth into the vat), inconsistent agitation (and thus amount of aeration) and not enough samples plated. Despite these problems the generation times obtained compare reasonably well with those predicted by the pseudomonad model (see bias and accuracy factors in Table 5.7). Figure 4.29 shows that there is a prolonged lag phase which appears to be approximately 2000 minutes. For the purpose of validations the duration of lag phase is required to

give an accurate estimate of the validity of the model. Without an accurate estimate of lag phase the predicted time will vary depending on the duration of lag phase, and will therefore influence the bias and accuracy factors. In this situation, the duration of the lag phase should be accurately determined a number of times so that the company microbiologists can input this information into '*Pseudomonas* Predictor' in order to use predictive modelling the most effectively.

5.5.4. 'Client' 4: Minced Beef

The data from client 4 at constant temperatures contains insufficient data points. Despite this the data shows a consistent trend, thereby allowing the generation time to be calculated manually.

Under fluctuating temperature regimes the predicted extent of growth was greater than that observed resulting in 'fail safe' situations. This is shown in Table 4.17 where the bias and accuracy factors range from 1.24 to 1.68 with a total bias of 1.39 and an accuracy of 1.42. While the degree of overprediction may be of some concern the lack of data on the duration of lag phase together with the natural variablity of viable count methods, which is usually considered to be ± 0.5 log cycles (Jarvis, 1989), would reduce the degree of error. Kamperman (1994) studied the effect of competitor microorganisms (*Brocothrix thermosphacta*, lactic acid bacteria, *Citrobacter freundii* and *Staphylococcus aureus*) on the growth rates of pseudomonads and found no interactive effects. The overprediction observed in Table 4.17 may also be due, in part, to excess carbon dioxide reducing the growth rate of the pseudomonads (Eyles *et al,* 1993, Willocx *et al,* 1993). This hypothesis may be studied by growing pseudomonads on thin slices of meat, rather than on minced beef, to determine whether the presence/absence of various gradients of carbon dioxide would affect the growth rates observed.

5.5.5. Comparison of Observed and Predicted Data

Bias and accuracy factors from all the 'clients' is shown in Table 5.7. Although the data has a total bias of 1.30 and an accuracy of 1.33 it can only be considered indicative as most of the data is not of a sufficient standard due to the inconsistencies in data collection. Despite this, and if the minced beef data are removed, the total bias and accuracy factors become 1.13 and 1.18 respectively (n=19), suggesting that '*Pseudomonas* Predictor' "works" (for dairy products) within the 20% error margin suggested by Walker & Jones (1994). The high bias and accuracy factors seen for the minced beef are possibly due to the presence of carbon dioxide in the beef samples. Further work is required to determine whether

gaseous atmosphere needs to be incorporated into the pseudomonad model for minced meat products.

Raw milk and cream were monitored under fluctuating temperature conditions. Table 5.7 shows that the pseudomonad model predicts well under these situations as bias and accuracy factors ranged from 1.12 to 1.14. This compares very well to results from the literature (Table 5.8) for fluctuating temperature conditions in which an overall bias and accuracy of 1.01 and 1.10, respectively, were found. Note that slightly improved bias and accuracy was found for the literature data than the data from industry and may be due to the lower level of control present in an industry situation. The accuracy factor of 1.10 for fluctuating temperature conditions in the laboratory was very similar to the accuracy of 1.09 obtained for growth experiments under constant temperature conditions in the laboratory (refer to 5.4.4.) suggesting that fluctuating temperatures do not greatly affect observed growth rates compared to those predicted by the model.

The temperature/water activity model for psychrotrophic pseudomonads predicts well at the temperature and water activity ranges tested. However, it should be noted that the product with the lowest water activity was that of evaporated milk (a_w=0.987) which does not have a a_w low enough to test the accuracy of the model at the lower limits of the a_w range. The model may be sufficiently accurate as no food product has been identified in which pseudomonads are the primary organism of concern at these low water activities.

Table 5.7: Bias and Accuracy Data for Industry Validation Trials.

Food Type	Bias	Accuracy	n
Raw Milk*	1.12	1.12	2
Reconstituted Whole Milk	1.11	1.21	10
UHT Milk	1.31	1.31	1
Cream*	1.14	1.14	6
Minced Beef*			
- at contant temperatures	1.06	1.06	2
- at fluctuating temperatures	1.39	1.42	43
TOTAL DATA	1.30	1.33	64

* = natural biota monitored

Table 5.8 Bias and accuracy factors of microorganisms under fluctuating temperature conditions (growth data and models obtained from the literature).

Author	Bias	Accuracy	n
Langeveld & Cuperus (1980)[1]	1.00	1.10	20
Mitchell *et al* (1995)[2]	0.99	1.12	8
Blankenship *et al* (1988)[3]	1.04	1.08	7
TOTAL DATA	1.01	1.10	35

1. *Enterobacter hafniae* B2 in milk under laboratory conditions
2. *Salmonella typhimurium* in broth under laboratory conditions
3. *Clostridium perfringens* in cooked chilli under laboratory conditions

5.6. INDUSTRY RESPONSE (QUESTIONNAIRE)

Many of the comments from respondents focussed on various problems with computer systems with many 'clients' feeling that '*Pseudomonas* Predictor' was needed in different spreadsheets (eg Quatropro) or versions of Lotus and Excel. The various industry participants had versions of Lotus and Excel from the very early editions right through to the latest available. When Excel 5 was released, *Pseudomonas* Predictor required complete rewriting as the Excel 4 document could not simply be upgraded to Excel 5 due to Microsoft changing the macro language. Given the number of problems encountered with computers during the evaluation process, it would appear that a specifically designed package would be a better alternative (rather than relying on an existing spreadsheets), as continual updates would then be unnecessary.

Validation trials require food products to be held at the worst conditions that can be found in industry in order to obtain the greatest extent of growth and therefore provide the greatest error if there is a problem with the models. Most microbiologists in the dairy industry are fully occupied with their normal work requirements during the milking season (December through to March) and yet this is the critical time regarding temperature control and validation studies. Later in the season, when the microbiologists have more time to do validation work, there

is relatively little milk and temperatures are cooler. It is also difficult to impress upon industry participants the requirement for large quantities of viable count data at all times (including nights and weekends). Combined, these factors suggest that in order to obtain satisfactory validation results in an industrial situation it is necessary for researchers to go out to industry and do the work themselves. Despite the low amount of interest in participating in validation trials 50% of 'clients' expressed interest in obtaining the validated '*Pseudomonas* Predictor' package and 72% were interested in similar packages for other microorganisms.

In many instances predictive modelling is still seen as a future technology and many industry correspondents are not ready for it yet. One respondent thought that 'in three or four years, shelf life and education of people along the cold chain will become much more important and I can see a role for the predictor there'. During the experimental evaluation process a similar attitude was found, indicating that perhaps many people in industry do not fully comprehend how to use predictive modelling and still see it as a technique for the future rather than as a current technology. This attitude may be hard to change as many people tend to be conservative when faced with the choice of carrying on using the old familiar methods or 'risking' a new technology (Sharpe, 1980). Once introduced, many quality initiatives fail because managers expect instant results when it can take several years to generate the 'culture' changes required for the new technology to work to its full potential (Sumner, 1995).

Many participants felt that although '*Pseudomonas* Predictor' made sense to them, as microbiologists, it needed to be designed differently so that it could be understood by non-microbiologists and would be user-friendly throughout the processing and distribution chain. In reality, it would be difficult to structure the software to be less technical which implies that either microbiologists monitor the entire processing and distribution chain or that food-handlers are educated to understand basic microbiological terms and concepts. Given that no 'hands-on' microbiology is required in order to use '*Pseudomonas* Predictor' the latter proposal would appear to be the most appropriate, particularly as many food-handlers might also require training for appropriate computer usage eg, how to setup/unload electronic temperature loggers for incorporation into the '*Pseudomonas* Predictor' program. Similarly many companies need to be approached about the potential uses of predictive microbiology and the advantages using such technology can provide eg cost/benefit analysis, incorporation into HACCP programs, product formulations and validity of regulations.

5.7. IMPROVEMENTS IN INDUSTRY-RESEARCH RELATIONSHIPS

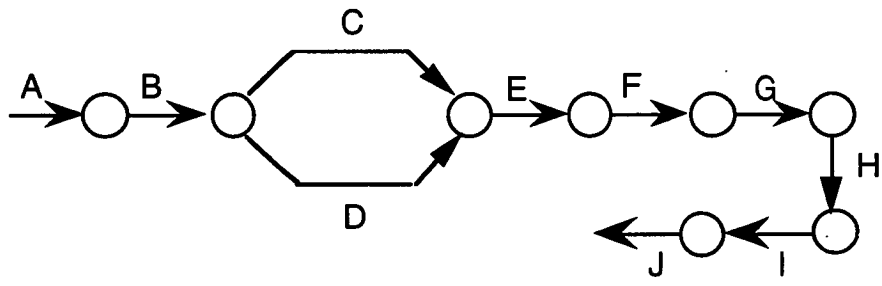
The problems discussed in developing and conducting industry trials for '*Pseudomonas* Predictor' could have been alleviated slightly had a different approach been utilised. This could best be described by using Network Analysis (Vitalis *et al*, 1987) in which a problem is broken down into a number of activities. In many respects it is similar to HACCP except that critical pathways rather than critical control points are identified. Each critical pathway is represented as an arrow with a circle defining the start of the activity and an arrow head defining the end of the activity as shown below. Each activity is identified by a letter or number.

The specific aims of the '*Pseudomonas* Predictor' program were to 1. develop a psychrotrophic pseudomonad model; 2. validate the pseudomonad model under constant and fluctuating temperature conditions; 3. to combine this information into interactive computer software for analysis of temperature history obtained by electronic data loggers. As the project progressed, the software and user's manual design were evaluated and the potential market assessed, resulting in another aim being (unofficially) added to the '*Pseudomonas* Predictor' program, this being to educate potential users of predictive modelling technology. The first step in utilising network analysis is to identify the particular activities (in no set order) required in order for these aims to reach successful completion. For the '*Pseudomonas* Predictor' program they could be described as

A Identification of pseudomonad strains of interest and the environmental conditions important to those strains
B Developing the pseudomonad models in broth systems
C Validation in food products in the laboratory
D Development of the software and users manual
E Obtain skills necessary to train potential users
F Validation trials of the pseudomonad model in industry
G Industry feedback on the software/manual design
H Collation of information and implementation of any changes
I Training for potential users
J Distribution.

In fact each of these steps could be broken down further, but the level of analysis is sufficient for this excercise. The purpose of network analysis is to structure the activities into a logical network so that the process is completed in the most efficient method and/or time possible. The '*Pseudomonas* Predictor' program was run in a more-or-less step-by-step fashion in which one activity was completed before another started, although to some extent 'C' and 'D' were run concurrently (Figure 5.5).

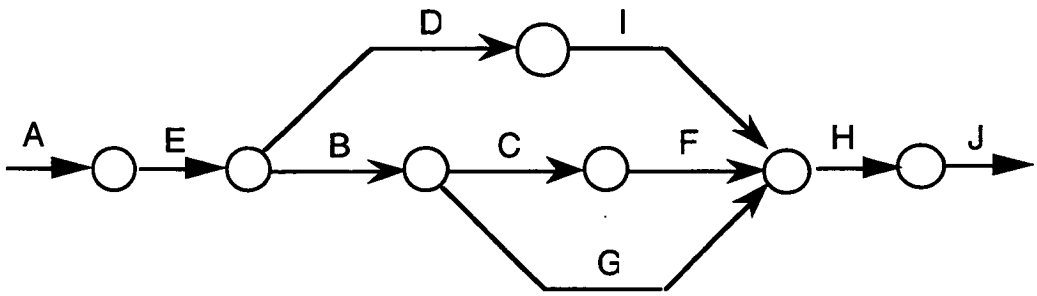
Figure 5.5 A network diagram showing how the '*Pseudomonas* Predictor' program was conducted.



The problem with this approach is that any problem or 'bottleneck' will hold up the entire program. The major 'bottlenecks' encountered consisted of problems such as a low rate of return of experimental data from industry complicated by the fact that even those that did industry trials often did not complete validation experiments as requested and many 'clients' exhibited a poor understanding of how best to utilise predictive microbiology methods. If the network analysis approach is used, the '*Pseudomonas* Predictor' program could have been reorganised as shown in Figure 5.6.

Using this approach, a 'mocked-up' version of the software and manual would be prepared so that step 'H' could start much earlier and perhaps be incorporated into a general training program (step 'I') teaching industry how to understand and apply predictive modelling techniques. Training (step 'I') occurred to a certain extent, with many talks and papers being presented to the Australian industry via venues such as the Australian Society for Microbiology (ASM) and the Australian Institute of Food Science and Technology (AIFST), however, because of the nature of these venues the information was aimed mostly at scientific personnel

Figure 5.6 A network diagram showing how the '*Pseudomonas* Predictor' program should have been conducted.



and so, in effect, we were 'talking to ourselves'. Unless training programs are directed at those who will use the product and at those who will enforce its use, through support at the managerial level, predictive microbiology will not be incorporated into industry. An example of this is provided by Bremner *et al* (1978), Olley & Thrower (1978) and Thrower *et al* (1978) who, almost 20 years ago, ran workshops on the effects of time-temperature relationships in fish handling and quality control at the Hawkesbury Agricultural College and at the Tasmanian College of Advanced Education. These courses had little impact on the fishing industry and their failure is almost certainly due to the format being inappropriate to potential users of the research. In effect many researchers and industry personnel are talking different languages. This barrier could be breached two ways;

1) Researchers should have some industry experience. Scientists often have little industry experience and thus have no idea of how a process really works, resulting in ideas that work in theory but are unrealistic in practice. Currently this is typified by students who start postgraduate studies as soon as they complete their undergraduate degree. This problem is being remedied through Industry Australian Postgraduate Awards which ensure that students works closely with industry and thus gains practical knowledge of their field of research.

2) ensure the person liaising with industry has the necessary skills to convey the information to potential users in a form they can understand, hence the importance of step 'E', in which the candidate attended 'Basic Training Skills' and 'Training Design and Evaluation' courses run by Training Services Australia.

Both methods result in the barrier between laboratory and factory being lessened resulting in more efficient communication and, hopefully, better implementation of research to industry.

As the model would not yet be validated (steps 'C' and 'F'), the training program must, of necessity, be restricted to a more general approach until the model had been developed (step 'B') and validations of the model were complete. By running the training program concurrently with the modelling program the amount of time available for teaching industry would be extended and therefore industry would be better prepared, mentally, for the application of the technology. In practice, this approach may not have worked as predictive microbiology had many critics at the commencement of the program, in the early 1990's, who would not have been prepared to learn about predictive modelling concepts until validation data was available thereby proving that the program could work. Similarly, at a managerial level, support for staff to attend training programs would not have been provided unless the managers could see a benefit to the company. No such benefit would be perceived by the managers while there was no proof of the viability of the modelling program.

In theory it was unnecessary to wait for the validations to be complete prior to starting development on the design of the software and manual. As Figure 5.6 shows, this could be started once the actual modelling is complete (step 'D') as long as the results from validations (steps 'C' and 'F') and industry feedback (step 'G') are incorporated into the final product (step 'H') before its release (step 'J'). In Figure 5.5, steps 'F' and 'G' were approached simultaneously with industry being sent software and manuals for '*Pseudomonas* Predictor' and asked to conduct validation trials. Experience showed that better industry validation data would have been obtained had the candidate targetted a couple of companies and personally undertaken the validations on site thereby separating steps 'F' and 'G' and allowing proportionally more time to be spent on step 'G' (as in Figure 5.6).

The network analysis approach has highlighted some important areas of concern in the implementation of '*Pseudomonas* Predictor' to industry, in particular, that although an optimal pathway was proposed in Figure 5.6 it was not possible to carry out due to the nature of the industry-research relationship. Industry needs to see some potential benefit in order to provide support for the implementation of a new technology, and while predictive microbiology remained theory, industry would not engage in wholesale training programs. Now that a validated model is available to industry, in the form of '*Pseudomonas* Predictor', thereby proving that predictive modelling can work, it is hoped that future modelling programs, for other microorganisms, may follow the pathway described in Figure 5.6 and be implemented in industry in the most efficient manner possible.

6. OUTCOMES

Predictive microbiology may be used to evaluate the bacteriological adequacy of a process or other operations in the preparation, distribution or storage of perishable foods. It cannot be used to assess the absolute bacteriological status of individual units in a system but will provide valuable comparative information on the loss of quality during any part of the processing chain or on the effect of changes in processing or distribution parameters or product formulation (Gill & Phillips, 1990).

The candidate has developed a model for psychrotrophic pseudomonads suitable for use where temperature and water activity are the main environmental parameters of concern. By developing the model using turbidimetric methods, prior to validations using viable count methods, large amounts of data were able to be collected resulting in a thorough understanding of the behaviour of pseudomonads under the conditions studied. For example, nearly 2200 observations (146 growth curves containing a minimum of 15 %T measurements each) were taken for the model for *P. putida* 1442. If models for the other pseudomonads and the validations are included over 8500 observations were recorded in laboratory experiments resulting in a highly validated and reliable model. Transfer of this technology to industry, in the form of '*Pseudomonas* Predictor', is currently proceeding.

The scope of operation of '*Pseudomonas* Predictor' is set by the operator who decides which part of the chain requires evaluation. The absolute limits i.e., acceptable starting and end points are also set by the operator on the basis of experience with the product and process or from HACCP or other quality assurance specifications. A number of general areas of use within a dairy processing and distribution chain were considered.

1) Pre-Factory:
'*Pseudomonas* Predictor' can be used to set specifications of initial psychrotrophic loads and temperature histories that will allow acceptable raw material to be received at the factory. These limits can be achieved by efficient hygiene, but '*Pseudomonas* Predictor' may be of use when isolating the cause of a problem (eg, where temperature control is breaking down) or as a learning tool. Farmers can be shown the quantitative, and thus financial, effect of why certain standards are enforced, or a particular process followed. For example, monitoring the milk prior to tanker pickup could be used to show farmers how cooling milk

just prior to collection (instead of refrigerating correctly from milking to pickup) will adversely effect the quality of their milk and thus the payment received.

'*Pseudomonas* Predictor' can be used to compare operating systems. For example, to show farmers the effect that an inefficient pre-cooling system may have on the quality of the raw milk. Improving the speed of cooling (i.e., upgrading the system or using the current system effectively) may decrease the extent of growth so that the milk falls into a higher price range (and the farmer is therefore paid a higher price for minimal effort). Alternatively, if a farmer wanted to upgrade his system '*Pseudomonas* Predictor' could determine whether the cost of upgrading is warranted.

2) Factory

'*Pseudomonas* Predictor' can be used to determine the effect of storage on raw milk in the silos. Based on growth predictions time/temperature limits could be imposed on the maximum time milk can remain in silos for use in a particular end product.

3) Factory to Factory Transfers

'*Pseudomonas* Predictor' can be used to determine whether a load of City Milk (ie, milk travelling from the main factory to a subsidary factory to pasteurise and package) should be accepted or rejected. This would be particularly important for milk loads travelling long distances. A time/temperature history together with information on the initial bacterial load, detected directly or set at a previously recorded maximum level, offers two potential benefits. The first, is that, if a tanker exceeded a set limit (i.e., incorporate the procedure as part of the HACCP program) a decision based on objective criteria, can be made regarding the suitability of that load for a particular end use. Secondly, tankers would not need to wait outside the recipient factory, while waiting for the test results to be cleared, before the milk can be downloaded into the silos. Downloading the temperature logger and determining the extent of *Pseudomonas* growth on arrival at the recipient factory would take no longer than 5 minutes. Apart from shortening the time until pasteurisation, this would improve relations with surrounding residences as some City Milk processors are sited in residential areas and during peak hours tankers wait outside the factory until the milk is cleared for use and can be downloaded.

4) Predictive modelling techniques can be used in the post pasteurisation storage and distribution systems. For example, incorporating '*Pseudomonas* Predictor' into the HACCP systems will allow identification of weak links in the processing and distribution chain. As on farm, '*Pseudomonas* Predictor' has potential as an education device.

Lag phase can be modelled under controlled laboratory conditions where the previous temperature histories is known (eg, in Chandler & McMeekin, 1985b). It is clear, however, that one of the main limiting factors of the pseudomonad model is that an understanding of how lag phase duration is affected by the history of the organism must be determined in order to minimise experiences such as those encountered in the industry validations where unexpectedly long lag phases were encountered. By being able to model lag phase, less product wastage would result and lag phase duration may be able to be increased by modifying the environmental conditions, thereby being of particular use in areas where regulating temperature control is difficult.

Users of the psychrotrophic pseudomonad model must remember what microorganism is relevant to their situation and the model must be used with care outside these regions. For example, pseudomonads may not be the dominant organism above 15°C. Thus, although the effect of temperature on the growth of pseudomonads can be determined, they may not necessarily be the dominant microbial group and thus the growth of other organisms such as mesophiles and thermodurics, may play a more important role.

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8. APPENDICES

8. APPENDICES

8.1. CULTURE MEDIA AND EQUIPMENT

8.1.1 CULTURE MEDIA

8.1.1.1. 0.1% Peptone

Bacteriological Peptone (Oxoid L37)	1.0 g
Distilled Water	1.0 L

Mix, dispense as required and sterilise (121°C/15 min)

8.1.1.2. Nutrient Broth No.2

Nutrient Broth No.2 (Oxoid CM67)	25 g
Distilled Water	1.0 L

Mix well, dispense as required and autoclave 121°C/15 min.

8.1.1.3. Pseudomonas Selective Agar (PSA)

Pseudomonas Agar Base (Oxoid CM559)	24.2 g
Glycerol (Unilab 243)	5.0 mL
Distilled Water	500 mL
Agar	2.0 g
CFC Selective Supplement (Oxoid SR 103)	1 vial
Sterile Distilled Water	2.0 mL

Mix together agar base, agar, water and glycerol. Boil to dissolve. Sterilise by autoclaving 121°C/15 min. Allow medium to cool to 50°C.
Rehydrate vial contents by adding 2 mL sterile distilled water. Add contents to cooled agar. Mix well and dispense as required.

8.1.1.4. Plate Count Agar (PCA)

Plate Count Agar Standard (APHA)(Oxoid CM 463)	23.5 g
Distilled Water	1.0 L

Boil, dispense as required and autoclave 121°C/15 min

8.1.1.5. Gram Stain

A suspension of bacteria was smeared onto a glass slide, allowed to dry and heat fixed. It was then flooded with crystal violet (1 minute), washed with water, flooded with iodine (1 minute), washed and decolourised with acetone/alcohol, washed and counterstained with safranin (1 minute), washed and allowed to dry.

Interpretation of results: Gram positive cells stain blue, while Gram negative cells stain red.

Crystal Violet

Crystal Violet	1.0 g
Distilled Water	100 mL

Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled Water	300 mL

Acetone/Alcohol

Acetone	100 mL
Alcohol	100 mL

Safranin

Safranin	1.0 g
Distilled Water	100 mL

8.1.1.6. Oxidase Reagent

NNNN-tetramethyl-*p*-phenylenediamine-dihydrochloride	1.0 g
Distilled Water	100 mL

Mix together. Soak strips of filter paper in reagent and air dry (in the dark) or freeze dry. Once dry, store in dark bottle below 4°C.

To test: Scrape some fresh young culture with a clean platinum wire (dirty or Nichrome wire may give a false positive result) and rub on the filter paper.

Interpretation of results: A blue colour within 10 seconds is positive. A blue colour in 10-60 seconds may be considered undeterminate.

(Kovacs, 1956; Cruikshank *et al*, 1975)

8.1.1.7. Oxidation/Fermentation using Hugh and Leifson Media

For Gram negative bacteria

Bacto Peptone (Oxoid L37)	2.0 g
NaCl (AnalaR Prod 10241 BDH)	5.0 g
K_2HPO_4 (Univar 2221)	0.3 g
Agar	3.0 g
Bromothymol Blue (1% alc. soln.)	3.0 mL
Glucose (Univar 783)	10.0 g
Distilled Water	1.0 L

Sterilise the glucose (dissolved in some of the distilled water) and the bromothymol blue using either filtration or momentary autoclaving to prevent carbohydrate decomposition which may occur during autoclaving. Momentary autoclaving involves turning the heat off as soon as the autoclave reaches 121°C and removing the contents as soon as possible (Cowan & Steel, 1974). Bring the remaining ingredients to the boil, pH to 6.9, autoclave 115°C / 20 min, allow to cool to 55°C and add the glucose and bromothymol blue solutions. Dispense into 10mL tubes.

To test: For each culture stab inoculate two tubes. Cover one with paraffin (anaerobic tube) and incubate both at 25°C/48 hours. If tubes have been stored, remelt agar and cool quickly (in ice) prior to inoculation.

Interpretation of results:

oxidative	= yellow at top of aerobic tube only
fermentative	= yellow in both tubes +/- gas
alkaline	= blue at top of aerobic tube only

8.1.1.8. Motility

Motility in isolates was tested using Method 1. In instances where motility was unclear, Method 2 was used.

Method 1:

The isolate was grown in Nutrient Broth No. 2 for 6 hours at the optimum temperature (30°C for pseudomonads). A small drop of the culture was placed on a microscope slide and observed using phase contrast. The presence or absence of motility was observed.

Method 2: (Cruikshank *et al*, 1975)

Agar	4.0 g Japanese agar *or*
	2.0 g New Zealand agar
Peptone Water (Oxoid CM9)	15.0 g
Distilled Water	1.0 L

Boil, dispense 5 mL into MaCartneys and autoclave 121°C/15 min.
To test: Stab inoculate into semi-solid agar. Incubate at the optimum temperature/2 days. Tubes should be checked after 24 hours.

Interpretation of results:

non-motile	growth confined to stab
motile	diffuse hazy growth

8.1.1.9. Nutrient Gelatin

'Lab-Lemco' Powder (Oxoid L29)	3.0 g
Peptone (Oxoid L37))	5.0 g
Gelatin (BBL 11868)	120.0 g
Distilled Water	1.0 L

Add gelatin to water and allow to stand for 15-30 minutes. Heat to dissolve gelatin, add and dissolve other ingredients. Adjust pH to 7.0 and distribute to test tubes. Autoclave at 115°C/20 min. Higher temperatures will result in gelatin not resolidifying. Dispense into 10mL tubes.

To test: Stab inoculate into tubes. Incubate at 25°C/2 days. If negative reincubate for up to 1 month. Before reading, place test tubes at 4°C for 1/2 hour to resolidify agar.

Interpretation of results:

-	no liquefaction	*P. fragi, P. putida*
+	minor liquefaction	
++	liquefaction	
+++	very strong liquefaction	*P. fluorescens*

8.1.1.10. King's B Medium for Fluorescein b

Proteose Peptone (Oxoid L46)	20.0 g
Glycerol (Unilab 243)	10.0 mL
K_2HPO_4 (Univar 2221)	1.5 g
$MgSO_4.7H_2O$ (Sigma No M-1880)	1.5 g
Agar	15.0 g
Deionised Water	1.0 L

Mix, adjust pH to 7.2 and autoclave at 121°C/12 min. Dispense as required.

To test: Streak plates using isolates to be tested. Incubate plates at 25°C/48 hr.

Interpretation of results: Place plates under UV lamp, at 254nm, and check for fluroescence(Cruikshank *et al*, 1975).

-	no fluroescence	*P. fragi*
+	minor fluroescence	
++	fluroescence	
+++	very strong fluroescence	*P. fluorescens, P. putida*

8.1.1.11. O/129 Sensitivity

Evenly inoculate the surface of an agar plate with the isolate to be tested. Place one 150µg disc (Oxoid DD15) on each plate. Incubate at optimum temperature for 24 hours.

Interpretation of results: (Lysenko, 1961)

Pseudomonas fragi	Sensitive
Ps. denitrificans	Sensitive
Ps. rubescens	Sensitive
All other *Pseudomonas* species	Resistant

8.1.1.12. Casein (Skim Milk) Agar

Skim Milk (made according to manufacturers instructions)	500 mL
Nutrient Agar, double strength	500 mL

Sterilise skim milk by autoclaving 115°C/10 min. Cool to 50°C. Sterilise nutrient agar by autoclaving 121°C/15 min. Cool to 50°C. Add to skim milk. Mix and dispense as required.
To test: Streak inculum onto plates and incubate plates at 25°C/2 days

Interpretation of results: plates were checked for zones of clearing.

-	no clearing	*P. fragi, P. putida*
+	minor clearing	
++	clearing	
+++	very strong clearing	*P. fluorescens*

8.1.1.13. Carbohydrate studies

Pseudomonads do not give reliable sugar reactions on peptone-containing media and should be grown on media with ammonium salt as the main N-source (Cowan & Steel, 1974).

Ammonium Salt Sugar (ASS)

$(NH_4)_2HPO_4$	1.0 g
KCl (BDH AnalaR Prod 10198)	0.2 g
$MgSO_4.7H_2O$ (BDH AnalaR Prod 10151)	0.2 g
Agar (Amyl Media RM 250)	20.0 g
Distilled Water	1.0 L
Bromothymol blue (0.2% solution)	4.0 mL

Add the solids to the water. Dissolve by steaming. Add bromothymol blue indicator and autoclave at115°C/20min. Cool to 60 °C. Add filter sterilised carbohydrate; glycerol (Unilab 243), xylose (Sigma X-1500), trehalose (Sigma T-5251), meso-inositol (Sigma No I-5125) to a final concentration of 1% and sucrose (Sigma S-5016) to a final concentration of 3%. Mix and distribute.

To test: Streak inculum onto plates and incubate plates at 25°C/2 days. The control is the isolate streaked onto ASS plates containing no carbohydrate.

Interpretation of results: All plates are compared to the strain on the control plate and rated on a scale of - (no growth) to +++.(very strong growth).

8.1.1.14. Maintenance of Culture at -70°C (Long Term Storage)

All cultures are maintained in duplicate. One is used for routine recovery, while the other is held in reserve.

Plastic (3mm) beads are washed in tap water with detergent, followed by dilute HCL to neutralise alkalinity. The beads are washed several times in tap water, then in distilled water and dried.

Approximately 20 beads are placed in each 2 mL screw-cap vial, which is then autoclaved at 121°C/15min

Bacteria are grown overnight on appropriate agar plates (Plate Count Agar) at the optimum temperature (30°C) for each bacterial strain. Approximately 1 mL of sterile (autoclave: 121°C/15min) 15% (v/v) glycerol in Nutrient Broth is pipetted onto the plate. Using a wire loop the growth is emulsified to make a thick suspension. The bacterial suspension is aseptically dispensed into two prepared vials. The suspension is aspirated several time to ensure the air bubbles inside the bead are displaced. Excess suspension is removed to prevent the beads sticking together when frozen. Vials are placed on their sides (to facilitate removal of beads when frozen) and stored in trays at -70°C.

To recover: one bead is removed and rubbed over the surface of a suitable solid medium and incubated appropriately. Bacteria will remain viable for at least 7 years.

8.1.2. EQUIPMENT

Analytical Profile Index (Api) 20 NE: kits (#2005) (API System, La Balme Les Grottes 38390 Montalieu Vercieu, France) used to identify non-enteric gram negative rods.

Balances: 1) Mettler PJ 3600 Delta Range®. Quoted precision ±0.01g. Mettler Instumente AG, Zurich, Switzerland. 2) MC1 Analytic AC210P (Sartorius Australia Pty Ltd, PO Box 84 Chadstone, Vic 3148, Aust). Quoted precision ±0.0001g.

Cricket Graph: a regression-fitting software package. Version 1.3.2. Cricket Software, Philadelphia, USA.

Electronic Temperature Loggers: Delphi loggers with a teflon freezer probe (MIRINZ, Hamilton, New Zealand). Quoted accuracy ±0.25°C over the operating range (-20°C to +40°C).

Fluke® Thermometer : Model 51K/J (John Fluke Manufacturing Co., Illinois, USA) electronic thermometer with Iron-Constantan thermocouple bead probe. Quoted accuracy: ±0.5°C. The calibration of the thermometer was routinely checked at 0°C and 100°C.

Gompertz function: SAS program to fit the Gompertz function, program written by Glen McPerson of the Mathematics Department, University of Tasmania, 1990.

L-tubes: test tubes containing a 90° bend resulting in an L-shaped tube of 40mL capacity.

pH meters: either 1) a Corning pH meter 120 (Corning Medical and Scientific, Scientific Instruments, Halstead, Essex, England) or 2) Orion Model 250A (portable) (Orion Research Inc, Boston, Mass., USA)

pH meter probes: either 1) Orion 91-06 Ag/AgCl probe (Orion Research Inc, Boston, Mass., USA) or 2) calomel sealed flat tip probe (AEP 433) Activon NSW. Australia.

Pipettors: A range of fixed and variable volume pipettors were used. These were
1) 'Fixopet': 100µl (fixed); 1 mL (fixed); 'Pluripet': 200 - 1000µl. Kartell Spa Via, Delle Industrie, 1 20082 Noviglio, Milan, Italy.
2) 'Pipetman': 200 - 1000µl. Gilson Medical Electronics (France) S.A., B.P. 45-95400 Villiers-le-Bel, France.
3) 'Oxford Macro-set': 5 - 10mL; 'Oxford Adjustable': 40 - 200µl. Oxford Laboratories, Inc., California. USA.

Spectrophotometer: Spectronic 20 (analogue display), Milton Roy Co., USA

Temperature Gradient Incubator: Model TN3. Advantec, Toyo Roshi International, California, USA

Timer: An electronic clock-timer (Model 870A, Jadco, China) was used to record time. At the time of inoculation, the timer was set to zero, and the real time recorded in case of timer failure.

UltraFit: A non-linear curve fitting package (Biosoft. PO Box 10938, Ferguson, MO 63135 USA).

Vortex Mixer: Model MT19 (Chiltern Scientific). Variable speed control from 300 to 2200 rpm.

Water activity measurement: Aqualab CX2 (Decagon Devices, Inc. PO Box 835, Pullman, Washington 99163, USA). Quoted accuracy ±0.003.

Waterbaths: A range of Lauda refrigerated waterbaths (Lauda DR.R. Wobser GMBH & Co. K.G., Lauda-Könighofen, West Germany) were used; Models RC20, RM20, M20, RM6 (R denoted refrigerated, the number indicates the bath capacity in litres).

APPENDIX 2: QUESTIONNAIRE
EVALUATION OF '*PSEUDOMONAS* PREDICTOR'

OPTIONAL	
Your Name:	_____
Your Position:	_____
Company:	_____
Address:	_____
	_____
Phone:	_____
NOTE: this information is confidential and will not be published.	

1a. In relation to *Pseudomonas* Predictor, did you

(i) look at the software package	Yes ☐ No ☐ (go to Q1c)
(ii) load the software onto your computer system?	Yes ☐ No ☐
(iii) familiarise yourself by using the dummy files provided?	Yes ☐ No ☐
(iv) set up your own files (temperature profiles)?	Yes ☐ No ☐
(v) carry out any validation trials?	Yes ☐ (go to Q1b) No ☐ (go to Q1c)

1b.

(i) What sort of validation trials were undertaken?

_____

_____

_____

_____

_____

_____

_____

(ii) Would you have used *Pseudomonas* Predictor more effectively if technical advice and/or support had been more readily available?

Yes ☐

No ☐

➪ Go to Q2

1c.

(i) Why not? (rank the relevant reasons)

☐ too busy with routine work

☐ dealing with urgent/unexpected problems

☐ wrong type of computer system

☐ too difficult to understand the manual/software

☐ personnel changes in your organisation

☐ not relevant to your situation

☐ other (please specify)_____

(ii) Would you have used *Pseudomonas* Predictor if technical support/advice had been more readily available?

Yes ☐

No ☐

➪ Go to Q2

2. Did you find any applications for *Pseudomonas* Predictor? Yes ❐ No ❐

If yes, please give brief details. _____

3. The **manual** is user-friendly. Do you:

Strongly agree ❐
Agree ❐
Neither agree or disagree ❐
Disagree ❐
Strongly disagree ❐

Are there any changes/improvements needed? Yes ❐ No ❐

If yes, what? _____

4. The **software** is user-friendly. Do you:

Strongly agree ❐
Agree ❐
Neither agree or disagree ❐
Disagree ❐
Strongly disagree ❐

Are there any changes/improvements needed? Yes ❐ No ❐

If yes, what? _____

5.

a) Would your organisation buy the finished version of *Pseudomonas* Predictor

Yes ❐ (go to Q5b)
No ❐ (go to Q6)

b) Similar products such as Food Micromodel (≈$2100) and the Delphi system (≈$600) are available. How much would your organisation be prepared to pay for *Pseudomonas* Predictor?

❐ $ 200 - 400
❐ $ 401 - 600
❐ $ 601+
❐ other (please specify) _____

6.

Do you consider that an extension officer should be employed to help users implement *Pseudomonas* Predictor in industry?

Yes ☐

No ☐

7.

a) Would you use predictive models for other organisms if they were available?

Yes ☐ (go to Q7b)

No ☐ (go to Q8)

b) what organisms and environmental factors would you like to see models developed for? _____

_____

8.

a) Do you think '*Pseudomonas* Predictor' is a suitable name for this product?

Yes ☐ (go to Q9)

No ☐ (go to Q8b)

b) Why not? _____

_____

9.

Do you have other comments?_____

_____

_____

_____

_____

_____

8.3. DATASETS USED FOR GENERATION OF PHSYCROTROPHIC PSEUDOMONAD MODELS

8.3.1. TEMPERATURE MODELS

8.3.1.1. Generation times of pseudomonad isolates in Nutrient Broth No. 2 at 10.8°C on the TGI (using %T data).

Isolate	Replicate				Av. GT(hrs)±SD
	1	2	3	4	
P.putida 119	4.09	4.25	4.26	4.35	4.24 ± 0.11
P.putida 1021	4.69	4.40	4.70	4.48	4.57 ± 0.15
P.putida 1261	3.83	3.94	4.49	4.44	4.17 ± 0.34
P.putida 1371	*	3.93	4.27	4.13	4.11 ± 0.17
P.fluorescens 1412	4.97	4.80	4.94	5.00	4.93 ± 0.09
P.fluorescens 1441	4.12	4.42	3.94	3.91	4.10 ± 0.23
P.putida 1442	3.42	3.20	3.40	3.27	3.32 ± 0.10
P.fluorescens 1451	4.50	4.33	4.00	3.94	4.19 ± 0.27
P.fluorescens 1681	4.17	3.89	3.59	3.66	3.83 ± 0.26
P.fragi ncimb 8542	3.38	3.33	3.19	3.17	3.27 ± 0.11
P.fluorescens I1	3.99	4.02	3.23	3.10	3.59 ± 0.49
P.putida I5	4.24	4.20	4.39	4.04	4.22 ± 0.14
P.fragi I6	3.77	3.58	3.51	3.28	3.53 ± 0.20
P.fluorescens I7	4.28	4.21	4.28	4.35	4.28 ± 0.06
P.putida I8.1	5.27	5.19	4.68	5.08	5.05 ± 0.26
P.fluorescens I8.2	3.78	3.81	4.03	4.15	3.94 ± 0.18
P.fluorescens I9	4.48	4.39	5.95	4.70	4.88 ± 0.73
P.fluorescens I10.1	4.04	4.08	4.31	4.07	4.12 ± 0.12
P.fluorescens I10.2	4.52	4.70	4.36	4.34	4.48 ± 0.17
P.putida I11	4.45	4.76	5.09	4.55	4.71 ± 0.28

* = no result due to breakage

8.3.1.2. Growth rates of *Pseudomonas putida* 1442 in Nutrient Broth No.2 at various temperatures on the TGI (expressed in terms of both %T and VC).

From 0 to 30°C

Temp (°C)	√r (%T)	√r (VC)
31.0	0.135	0.165
30.0	0.133	0.163
28.9	0.133	0.163
28.2	0.131	0.161
27.4	0.131	0.160
26.5	0.129	0.157
25.8	0.125	0.153
25.0	0.126	0.154
24.3	0.121	0.148
23.5	0.119	0.146
22.8	0.117	0.144
22.1	0.109	0.133
21.3	0.109	0.134
20.6	0.107	0.131
19.8	0.104	0.127
19.0	0.102	0.124
18.3	0.097	0.119
17.3	0.095	0.116
16.4	0.090	0.110
15.6	0.088	0.108
14.6	0.083	0.102
13.7	0.080	0.098
12.7	0.075	0.092
11.9	0.071	0.087
10.1	0.068	0.083
9.1	0.060	0.074
8.3	0.054	0.066
5.2	0.051	0.063
4.0	0.046	0.056
0.7	0.036	0.044

From 0 to 15°C

Temp (°C)	√r (%T)	√r (VC)
15.2	0.088	0.107
14.8	0.084	0.103
14.3	0.082	0.101
13.8	0.082	0.101
13.3	0.081	0.099
12.9	0.075	0.092
12.7	0.076	0.093
12.2	0.076	0.093
11.9	0.076	0.093
11.2	0.071	0.087
10.7	0.070	0.086
10.3	0.069	0.085
10.0	0.067	0.081
9.5	0.067	0.082
8.9	0.064	0.078
8.6	0.059	0.072
8.2	0.059	0.073
7.7	0.056	0.069
7.2	0.056	0.069
6.0	0.050	0.062
5.3	0.048	0.058
4.9	0.045	0.055
4.2	0.042	0.051
3.2	0.042	0.051
2.7	0.038	0.047
2.2	0.037	0.046
1.2	0.034	0.042
0.2	0.031	0.038
-0.4	0.027	0.033

From 20 to 50°C

Temp (°C)	√r (%T)	√r (VC)
46.4	no growth	no growth
44.1	no growth	no growth
42.1	no growth	no growth
40.5	no growth	no growth
39.2	0.069	0.084
37.6	0.073	0.089
36.1	0.097	0.119
34.8	0.102	0.124
33.7	0.103	0.126
32.6	0.130	0.159
32.1	0.136	0.166
31.2	0.136	0.166
30.0	0.135	0.165
29.2	0.131	0.160
28.5	0.133	0.163
28.0	0.132	0.161
27.2	0.131	0.160
26.6	0.131	0.160
26.0	0.128	0.157
25.4	0.127	0.155
24.7	0.121	0.148
24.1	0.124	0.152
23.5	0.123	0.151
23.1	0.121	0.148
22.2	0.115	0.141
21.7	0.111	0.136
21.0	0.111	0.136
20.4	0.114	0.139
20.0	0.108	0.132
19.4	0.108	0.132

8.3.1.3. Growth rates of a *Pseudomonas* cocktail (5 strains, consisting of *P.putida* 1442, *P.fragi* I6, *P.fluorescens* 1412, *P.fluorescens* I1 and *P.fluorescens* I8.2) in Nutrient Broth No.2 at various temperatures (expressed in terms of both %T and VC)

3.3.1. From 0 to 30°C

Temp (°C)	√r (%T)	√r (VC)
31.0	0.126	0.155
30.1	0.133	0.163
29.1	0.131	0.160
28.2	0.130	0.159
27.2	0.128	0.156
26.5	0.128	0.156
25.8	0.123	0.150
25.0	0.120	0.147
24.2	0.115	0.140
23.4	0.113	0.138
22.7	0.109	0.133
22.0	0.106	0.130
21.3	0.103	0.126
20.6	0.100	0.123
19.8	0.094	0.116
19.0	0.095	0.116
18.3	0.088	0.108
17.3	0.087	0.107
16.4	0.084	0.103
15.6	0.079	0.097
14.6	0.078	0.096
13.7	0.074	0.091
12.7	0.070	0.086
11.9	0.065	0.079
9.9	0.060	0.073
9.1	0.056	0.069
8.3	0.052	0.064
5.3	0.047	0.058
4.0	0.043	0.052
0.9	0.036	0.044

From 0 to 15°C

Temp (°C)	√r (%T)	√r (VC)
14.9	0.089	0.109
14.5	0.084	0.103
14.0	0.080	0.098
13.5	0.081	0.100
13.1	0.080	0.098
12.6	0.079	0.097
12.3	0.078	0.095
11.9	0.074	0.091
11.5	0.074	0.091
11.2	0.072	0.088
10.8	0.070	0.085
10.4	0.068	0.083
9.9	0.064	0.079
9.6	0.063	0.077
9.0	0.062	0.076
8.4	0.058	0.071
8.1	0.056	0.069
6.8	0.054	0.066
6.1	0.049	0.060
5.3	0.049	0.060
4.8	0.048	0.058
4.2	0.045	0.055
3.6	0.041	0.050
2.9	0.041	0.050
2.2	0.038	0.047
1.7	0.037	0.045
0.6	0.031	0.038

8.3.1.4. Growth rates of *Pseudomonas fragi* NCIMB 8542 in Nutrient Broth No.2 at various temperatures on the TGI (expressed in terms of both %T and VC).

From 0 to 30°C (Experiment 1)

Temp (°C)	√r (%T)	√r (VC)
26.7	0.121	0.148
26.0	0.118	0.144
25.1	0.119	0.146
24.1	0.119	0.146
23.4	0.118	0.144
22.8	0.113	0.138
22.1	0.113	0.138
21.4	0.107	0.131
20.8	0.108	0.132
20.1	0.106	0.130
19.5	0.102	0.125
18.9	0.115	0.141
18.3	0.098	0.121
17.5	0.096	0.118
17.0	0.094	0.115
16.1	0.092	0.113
15.5	0.088	0.108
14.7	0.084	0.103
13.9	0.080	0.098
13.1	0.078	0.095
12.2	0.075	0.092
11.4	0.071	0.087
10.5	0.066	0.081
9.5	0.062	0.076
8.3	0.059	0.073
6.8	0.055	0.067
5.7	0.048	0.059
4.4	0.043	0.053
3.2	0.037	0.045
1.6	0.032	0.039

From 0 to 30°C (Experiment 2)

Temp (°C)	√r (%T)	√r (VC)
26.4	0.112	0.137
25.8	0.111	0.136
24.9	0.108	0.133
24.0	0.105	0.129
23.2	0.104	0.128
22.6	0.101	0.123
21.9	0.099	0.121
21.3	0.098	0.120
20.6	0.098	0.120
19.8	0.092	0.112
19.2	0.090	0.111
18.5	0.089	0.108
18.1	0.088	0.107
17.3	0.082	0.101
16.6	0.082	0.101
16.0	0.078	0.096
15.3	0.078	0.095
14.6	0.074	0.091
13.8	0.071	0.087
12.9	0.069	0.084
12.1	0.068	0.083
11.2	0.061	0.074
10.3	0.057	0.070
9.3	0.054	0.066
7.7	0.051	0.062
6.4	0.045	0.055
4.8	0.042	0.052
3.5	0.038	0.047
2.1	0.032	0.039
0.5	0.027	0.033

8.3.1.5. Growth rates of *Pseudomonas fragi* I6 in Nutrient Broth No.2 at various temperatures on the TGI (expressed in terms of both %T and VC)

From 0 to 30°C (Experiment 1)

Temp (°C)	√r (%T)	√r (VC)
26.6	0.132	0.161
25.6	0.130	0.159
24.7	0.123	0.151
24.2	0.123	0.151
23.5	0.120	0.147
22.6	0.116	0.143
22.1	0.114	0.140
21.3	0.110	0.135
20.7	0.110	0.134
20.1	0.106	0.130
19.5	0.102	0.125
18.8	0.098	0.120
18.2	0.098	0.120
17.5	0.096	0.117
16.9	0.093	0.114
16.2	0.089	0.109
15.4	0.084	0.103
14.6	0.085	0.104
13.9	0.079	0.097
13.2	0.075	0.092
12.2	0.073	0.090
11.4	0.071	0.086
10.5	0.064	0.079
9.4	0.060	0.073
8.3	0.056	0.069
7.3	0.050	0.061
5.8	0.046	0.056
4.8	0.040	0.049
3.6	0.038	0.047
1.9	0.029	0.035

From 0 to 30°C (Experiment 2)

Temp (°C)	√r (%T)	√r (VC)
26.5	0.113	0.138
25.6	0.115	0.141
24.6	0.112	0.137
23.9	0.105	0.129
23.3	0.105	0.129
22.6	0.102	0.125
21.8	0.101	0.124
21.1	0.099	0.122
20.5	0.096	0.118
19.8	0.094	0.115
19.1	0.094	0.115
18.5	0.089	0.109
18.0	0.088	0.108
17.2	0.085	0.105
16.6	0.084	0.103
15.8	0.079	0.097
15.1	0.078	0.096
14.2	0.071	0.087
13.6	0.068	0.083
12.7	0.070	0.086
12.0	0.066	0.081
11.1	0.059	0.072
10.3	0.055	0.067
8.9	0.051	0.063
7.6	0.048	0.058
6.7	0.044	0.054
5.3	0.041	0.050
3.5	0.033	0.040
2.1	0.036	0.044
0.5	0.024	0.030

From 0 to 30°C (Experiment 3)

Temp (%T)	√r (%T)	√r (VC)
29.1	0.123	0.151
28.1	0.119	0.146
26.9	0.123	0.150
26.0	0.120	0.147
25.0	0.115	0.141
24.4	0.111	0.136
23.6	0.113	0.138
22.7	0.107	0.131
21.9	0.105	0.129
21.2	0.100	0.122
20.5	0.100	0.123
19.8	0.096	0.118
19.1	0.095	0.117
18.4	0.090	0.111
17.6	0.085	0.104
16.8	0.086	0.106
15.9	0.078	0.095
15.2	0.077	0.094
14.3	0.074	0.091
13.5	0.071	0.087
12.7	0.071	0.087
11.7	0.064	0.079
10.6	0.059	0.072
9.6	0.055	0.067
8.3	0.051	0.063
6.8	0.046	0.057
5.1	0.040	0.048
3.4	0.035	0.043
2.3	0.033	0.040
0.4	0.025	0.030

8.3.1.6. Growth rates of *Pseudomonas fluorescens* 1412 in Nutrient Broth No.2 at various temperatures on the TGI (expressed in terms of both %T and VC)

3.6.1. From 0 to 30°C

Temp (°C)	√r (%T)	√r (VC)
29.8	0.117	0.144
28.6	0.124	0.152
27.5	0.120	0.147
26.2	0.120	0.147
25.1	0.114	0.140
24.3	0.114	0.139
23.5	0.107	0.131
22.6	0.107	0.131
21.8	0.107	0.131
21.0	0.096	0.117
19.9	0.100	0.123
19.3	0.096	0.118
18.5	0.095	0.117
17.6	0.089	0.109
16.8	0.087	0.106
16.2	0.082	0.101
15.2	0.079	0.097
14.4	0.079	0.097
13.6	0.074	0.090
12.7	0.067	0.083
11.8	0.067	0.082
10.9	0.066	0.081
9.7	0.060	0.073
8.8	0.055	0.067
7.4	0.051	0.062
5.8	0.049	0.060
4.2	0.043	0.053
2.6	0.036	0.044
1.3	0.032	0.039
-0.6	0.030	0.037

From 20 - 50°C

Temp (°C)	√r (%T)	√r (VC)
47.1	no growth	no growth
45.2	no growth	no growth
42.7	no growth	no growth
40.9	no growth	no growth
38.9	no growth	no growth
37.7	no growth	no growth
36.5	no growth	no growth
35.4	0.055	0.067
34.1	0.076	0.093
32.9	0.104	0.127
32.1	0.116	0.142
31.2	0.122	0.149
30.4	0.126	0.154
29.5	0.125	0.153
28.5	0.122	0.149
27.9	0.124	0.152
27.2	0.123	0.151
26.4	0.114	0.139
26.0	0.119	0.146
25.2	0.113	0.138
24.7	0.095	0.117
24.0	0.107	0.131
23.4	0.099	0.121
22.8	0.105	0.129
22.1	0.108	0.132
21.5	0.102	0.125
20.8	0.094	0.116
20.4	0.095	0.117
20.0	0.093	0.114
19.5	0.088	0.107

8.3.2. DATASETS USED FOR GENERATION OF PSYCHROTROPHIC PSEUDOMONAD WATER ACTIVITY MODELS

8.3.2.1. Growth of *P.putida* 1442 in Nutrient Broth No.2 at various water activities at 20°C (using %T data).

a_w	Replicate		Av GT(min)	r
	1	2		(1/GT)
0.995	87.89	86.82	87.35	0.0114
0.994	101.23	83.12	92.18	0.0108
0.993	92.02	80.93	86.47	0.0116
0.990	87.92	87.00	87.46	0.0114
0.988	86.61	95.39	91.00	0.0110
0.987	104.38	97.74	101.06	0.0099
0.984	112.21	112.07	112.14	0.0089
0.983	116.38	112.69	114.54	0.0087
0.981	129.43	116.36	122.89	0.0081
0.978	133.98	133.69	133.84	0.0075
0.976	131.34	147.06	139.20	0.0072
0.975	180.38	151.82	166.10	0.0060
0.972	181.49	179.07	180.28	0.0055
0.970	192.59	174.21	183.40	0.0055
0.968	222.51	216.02	219.27	0.0046
0.965	225.95	299.89	262.92	0.0038
0.963	215.75	285.44	250.59	0.0040
0.961	243.80	319.23	281.51	0.0036
0.958	298.38	446.50	372.44	0.0027
0.956	412.19	557.57	484.88	0.0021
0.954	685.86	719.16	702.51	0.0014
0.951	1055.76	1133.71	1094.73	0.0009
0.949	1715.82	1943.58	1829.70	0.0005
0.947	3112.74	2979.89	3046.31	0.0003
0.990	96.84	91.82	94.33	0.0106
0.978	127.89	123.28	125.58	0.0080
0.968	205.57	214.90	210.24	0.0048
0.955	722.97	789.83	756.40	0.0013

8.3.2.2. Growth of *P.fragi* 8542 in Nutrient Broth No.2 at various water activities at 20°C (using %T data).

a_w	Replicate		Av GT(min)	r
	1	2		(1/GT)
0.995	92.89	89.94	91.41	0.0109
0.993	94.65	98.23	96.44	0.0104
0.990	94.66	107.13	100.89	0.0099
0.987	106.54	109.50	108.02	0.0093
0.984	113.70	126.86	120.28	0.0083
0.981	129.68	137.12	133.40	0.0075
0.978	141.66	143.46	142.56	0.0070
0.975	177.40	179.86	178.63	0.0056
0.972	176.45	168.16	172.31	0.0058
0.968	194.35	212.56	203.46	0.0049
0.965	279.03	278.53	278.78	0.0036
0.962	366.75	361.21	363.98	0.0027
0.958	642.86	722.35	682.61	0.0015
0.955	1094.22	1251.57	1172.90	0.0009

8.3.2.3. Growth of *P.fluorescens* 1412 in Nutrient Broth No.2 at various water activities at 20°C (using %T data).

a_w	Replicate		Av GT(min)	r
	1	2		(1/GT)
0.995	92.89	91.40	92.14	0.0109
0.993	103.11	107.80	105.46	0.0095
0.990	117.84	115.13	116.49	0.0086
0.987	133.25	119.70	126.48	0.0079
0.984	125.59	135.82	130.71	0.0077
0.981	157.29	142.29	149.79	0.0067
0.978	167.64	177.20	172.42	0.0058
0.975	198.97	193.37	196.17	0.0051
0.972	318.83	252.64	285.74	0.0035
0.968	329.16	329.88	329.52	0.0030
0.965	472.32	518.66	495.49	0.0020
0.962	767.77	750.40	759.08	0.0013
0.958	1550.19	1334.58	1442.38	0.0007
0.955	3133.45	2287.99	2710.72	0.0004
0.951	3039.17	6496.89	4768.03	0.0002

8.3.2.4. Growth of *P.putida* 1261 in Nutrient Broth No.2 at various water activities at 20°C (using %T data).

a_w	Replicate GTs		Av GT(min)	r
	1	2		(1/GT)
0.995	106.69	116.29	111.49	0.0090
0.993	126.86	114.78	120.82	0.0083
0.990	118.33	121.80	120.06	0.0083
0.987	126.37	128.22	127.30	0.0079
0.984	155.98	140.63	148.30	0.0067
0.981	158.00	169.31	163.65	0.0061
0.978	174.47	173.55	174.01	0.0057
0.975	214.70	204.43	209.57	0.0048
0.972	220.87	205.49	213.18	0.0047
0.968	252.55	270.20	261.38	0.0038
0.965	295.30	349.93	322.61	0.0031
0.962	457.04	458.01	457.52	0.0022
0.958	743.67	625.64	684.65	0.0015
0.955	1024.38	1026.23	1025.30	0.0010
0.951	2497.43	2506.66	2502.04	0.0004
0.947	13664.70	13500.34	13582.52	0.0001

8.3.2.5. Growth of *P.fluorescens* 8.2 in Nutrient Broth No.2 at various water activities at 20°C (using %T data).

a_w	Replicate GTs		Av GT(min)	r
	1	2		(1/GT)
0.995	88.659	78.842	83.751	0.0119
0.993	94.623	96.870	95.747	0.0104
0.990	103.162	96.793	99.978	0.0100
0.987	111.156	110.615	110.886	0.0090
0.984	124.398	127.643	126.020	0.0079
0.981	138.868	139.655	139.261	0.0072
0.978	166.372	163.575	164.973	0.0061
0.975	198.326	192.764	195.545	0.0051
0.972	225.978	212.957	219.468	0.0046
0.969	276.710	291.078	283.894	0.0035
0.965	369.526	329.241	349.383	0.0029
0.962	538.323	481.812	510.067	0.0020
0.959	672.034	595.391	633.712	0.0016
0.955	865.945	977.684	921.814	0.0011
0.951	1960.723	1486.252	1723.487	0.0006
0.948	4495.837	3846.253	4171.045	0.0002

8.3.3. COMBINED TEMPERATURE / WATER ACTIVITY MODELS

8.3.3.1 *P. putida* 1442 Dataset expressed in terms of %T

a_w	Temp (°C)	GT (min)	√r (1/min)
0.996	26.8	41.4	0.155
	23.2	44.2	0.150
	19.6	57.8	0.131
	15.7	80.4	0.112
	10.8	134.3	0.086
	2.6	433.3	0.048
0.977	27.5	66.4	0.123
	23.9	80.6	0.111
	20.4	93.1	0.104
	16.5	107.3	0.097
	11.8	134.9	0.086
	3.9	471.0	0.046
0.969	28.3	122.6	0.090
	24.5	119.2	0.092
	21.0	126.8	0.089
	17.2	130.5	0.088
	12.8	238.7	0.065
	5.5	659.3	0.039
0.960	29.0	507.8	0.044
	25.1	286.7	0.059
	21.5	303.8	0.057
	17.9	350.7	0.053
	13.4	520.7	0.044
	7.3	1716.5	0.024

8.3.3.2 *P. fluorescens* 1412 dataset expressed in terms of %T

a_w	Temp (°C)	GT (min)	√r (1/min)
0.996	26.3	50.1	0.141
	22.9	58.4	0.131
	19.4	78.2	0.113
	15.5	120.0	0.091
	10.7	169.9	0.077
	2.1	548.1	0.043
0.977	26.8	122.8	0.090
	23.5	105.8	0.097
	20.1	107.9	0.096
	16.2	132.7	0.087
	11.3	207.3	0.069
	4.1	659.9	0.039
0.969	27.9	242.0	0.064
	24.1	204.5	0.070
	20.7	200.5	0.071
	16.8	221.3	0.067
	12.3	418.3	0.049
	5.5	889.3	0.034
0.960	28.8	1054.8	0.031
	24.9	993.4	0.032
	21.2	866.4	0.034
	17.5	1024.0	0.031
	13.4	1007.3	0.032
	7.4	2788.9	0.019

8.3.4. GROWTH RATES OF *P. PUTIDA* 1442 IN NUTRIENT BROTH NO.2 AT VARIOUS pH AT 20°C (USING %T DATA).

pH	r (1/min)
7.74	0.0112
7.57	0.0098
7.52	0.0102
7.04	0.0098
6.51	0.0095
6.12	0.0090
5.81	0.0079
5.66	0.0072
5.53	0.0074
5.41	0.0077
5.27	no growth
5.21	no growth
5.09	no growth
5.02	no growth
4.88	no growth
7.79	0.0106
7.60	0.0100
7.40	0.0102
7.07	0.0098
6.61	0.0096
6.13	0.0087
5.83	0.0066
5.69	0.0066
5.54	0.0083
5.42	0.0076
5.26	no growth
5.09	no growth
5.04	no growth
4.89	no growth

84. GROWTH RATE STUDIES IN PRODUCT (LABORATORY)

8.4.1. MILK

8.4.1.1. Growth rates of *P.putida* 1442 in modified milk under minimal aeration conditions

Temperature (°C)	$\sqrt{r}$	
	observed	predicted
25.0	0.138	0.150
25.0	0.146	0.150
25.0	0.147	0.150
15.0	0.088	0.104
10.0	0.074	0.081
10.0	0.072	0.081
8.0	0.072	0.072
8.0	0.069	0.072
4.0	0.050	0.054
2.0	0.038	0.045
2.0	0.042	0.045

8.4.1.2. Growth rates of *P.putida* 1442 in modified milk (optimal conditions)

Temperature (°C)	√r (VC) observed	√r (VC) predicted	Log cfu/mL when pH=6.5
26.5	0.161	0.157	
26.5	0.161	0.157	
26.6	0.154	0.158	
26.6	0.157	0.158	
12.9	0.100	0.095	
12.9	0.100	0.095	
12.9	0.102	0.095	
12.9	0.100	0.095	
3.1	0.047	0.050	
3.1	0.048	0.050	
3.1	0.049	0.050	
3.1	0.049	0.050	
9.9	0.082	0.081	
10.0	0.081	0.081	
8.2	0.070	0.073	
8.1	0.072	0.073	
6.2	0.065	0.064	
6.2	0.064	0.064	
4.2	0.055	0.055	
4.1	0.055	0.055	
2.1	0.042	0.045	
1.8	0.042	0.044	
0.1	0.038	0.036	
0.1	0.038	0.036	
-1.1	0.035	0.030	
21.3	0.134	0.134	9.70
17.6	0.115	0.116	9.68
14.0	0.101	0.100	9.05
12.1	0.094	0.091	9.01
10.4	0.083	0.083	9.02
7.1	0.067	0.068	9.00
5.2	0.062	0.059	8.80
3.9	0.053	0.053	8.77
2.8	0.051	0.049	8.84

8.4.1.3. Growth rates of *P. putida* 1442 in whole milk (optimal conditions)

Temperature (°C)	√r (VC) observed	√r (VC) predicted	Log cfu/mL when pH=6.5
21.2	0.134	0.133	9.70
17.7	0.119	0.117	9.40
13.8	0.090	0.099	9.51
12.3	0.088	0.092	9.41
10.5	0.078	0.084	8.80
7.1	0.068	0.068	8.85
5.7	0.060	0.062	8.70
3.9	0.055	0.053	8.70
2.8	0.050	0.049	8.50
26.1	0.169	0.155	
26.9	0.160	0.159	
16.5	0.116	0.111	
16.4	0.113	0.111	
25.7	0.152	0.153	9.90
26.4	0.151	0.157	9.90
21.2	0.137	0.133	9.20
21.2	0.135	0.133	9.30
17.4	0.121	0.115	9.15
17.4	0.119	0.115	9.20
14.4	0.110	0.102	9.10
14.5	0.110	0.102	9.10

8.4.1.4. Growth rates of pseudomonads in uninoculated raw milk (optimal conditions)

Temperature (°C)	√r	
	observed	predicted
23.8	0.137	0.145
18.6	0.118	0.121
13.6	0.083	0.098
9.8	0.070	0.080
4.9	0.054	0.058
1.0	0.038	0.040
19.9	0.134	0.127
16.1	0.115	0.110
12.7	0.098	0.094
7.5	0.070	0.070
4.5	0.058	0.056
2.8	0.049	0.049
0.3	0.040	0.037

8.4.2. EVAPORATED MILK

Temp (°C)	√r (1/min) observed (PCA)	√r (1/min) observed (PSA)	√r (1/min) predicted
26.3	0.183		0.138
25.6	0.176		0.137
23.4	0.147		0.130
22.7	0.177		0.128
20.7	0.153		0.121
19.4	0.107		0.116
16.4	0.107		0.103
12.8	0.090		0.089
10.3	0.076		0.078
8.0	0.062		0.068
6.2	0.057		0.060
25.9	0.190	0.141	0.141
22.4	0.195	0.138	0.129
18.5	0.120	0.115	0.114
16.1	0.102	0.101	0.104
9.5	0.076	0.075	0.076
2.8	0.049	0.049	0.046

8.4.3. CREAM

Brand	Temp (°C)	√r (1/min) observed PCA	√r (1/min) observed PSA	√r (1/min) predicted
1	15	0.092	not detected	
	11	0.066	0.084	0.088
	6	0.057	0.059	0.065
	2	0.041	0.041	0.046
2	11	0.077		0.088
	6	0.064		0.065
	2	0.049		0.046

8.5. GROWTH RATE STUDIES IN PRODUCT (INDUSTRY)

8.5.1. 'CLIENT' 1: RAW MILK

Farm 1

Trial 1: no colonies were observed on the dilutions plated

Trial 2:

Milking 1

Time (min)	Log cfu/mL
0	0.84
120	0.74
239	0.65
365	0.40
487	1.02

Milking 2

Time (min)	Log cfu/mL
0	0.85
66	1.15
124	1.34
185	0.60
235	1.36

Farm 2

Trial 1: no colonies were observed on the dilutions plated

Trial 2:

Time (min)	Log cfu/mL
0	1.23
58	0.48
120	0.70
169	0.48

Farm 3

Trial 1

Milking 1

Time (min)	Log cfu/mL
0	2.94
459	3.26

Milking 2

Time (min)	Log cfu/mL
0	2.61
152	2.63
293	2.59
349	2.59
603	2.54
1084	3.24

Milking 3

Time (min)	Log cfu/mL
0	2.76
49	2.93
120	2.78
176	2.53

Trial 2

Milking 1

Time (min)	Log cfu/mL
0	2.71
513	2.78

Milking 2

Time (min)	Log cfu/mL
0	2.48
120	2.48
247	2.51
414	2.55
824	2.48
1257	2.50

8.5.2. 'CLIENT' 2: RECONSTITUTED WHOLE MILK AND UHT MILK

Strain 1 in UHT milk at 15°C

Time (days)	Log cfu/mL
0	1.77
1	5.14
2	7.96
3	9.91
4	10.07
6	10.21

Reconstituted Whole Milk at 7°C

Time (days)	Log cfu/mL				
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
0	2.11	2.04	2.08	2.00	1.78
1	3.07	2.84	2.90	3.07	2.80
2	4.03	3.89	4.51	4.84	3.83
3	5.45	5.47	5.89	6.83	5.55
4	6.74	6.93	7.14	7.95	6.81
5	7.18	7.26	7.34	7.00	7.43
6	7.28	7.48	7.78		7.51

Reconstituted Whole Milk at 4°C

Time (days)	Log cfu/mL				
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
0	2.00	1.60	1.78	2.28	1.48
1	2.83	2.60	2.76	2.89	2.04
2	3.80	3.66	3.61	3.81	2.30
3	4.98	4.90	4.86	5.63	3.64
4	6.07	6.10	6.20	7.07	5.21
5	6.90	6.89	6.94	7.52	6.12
6	7.72	7.38	7.43	7.71	6.95
7	7.54	7.79	7.61		7.46

8.5.3. 'CLIENT' 3: CREAM

Time (min)	Log cfu/mL	
	Total Viable	Pseudomonad
0	1.30	1
970	1	1
1235	1.18	1
1450	1.30	1
2400	2	1.3
2670	2.18	1
2880	2.48	1.3
3840	4.76	4.15
4095	4.83	4.6
4320	4.01	4.82

8.5.4. 'CLIENT' 4: MINCED BEEF

8.5.4.1. Constant Temperature Conditions

2.2°C	
Time (min)	Log cfu/mL
0	3.86
1440	4.61
2880	5.55
4200	6.36
5610	7.19
7110	8.01
8640	8.73

2.3°C	
Time (min)	Log cfu/mL
0	4.62
1440	5.58
2880	6.47
4320	7.40

8.5.4.2. Fluctuating Temperature Conditions

Trial #1

Time (min)	Log cfu/mL
0	3.60
1000	3.54
1410	3.56
2455	5.32
2870	5.75
3880	6.28
4310	6.56
5320	7.00
5735	7.08

Trial #2

Time (min)	Log cfu/mL
0	3.41
465	3.30
1425	3.41
1845	3.60
2875	5.67
3285	5.69
4320	6.32
4725	6.63
5745	6.99
6180	7.30

Trial #3

Time (min)	Log cfu/mL
0	3.49
970	3.67
1220	3.89
1460	3.89
2430	5.51
2665	5.40
2900	5.43
3865	5.61
4100	5.72
4380	5.64
5305	5.99
5540	6.15
5850	6.08

Trial #4

Time (min)	Log cfu/mL
0	3.51
960	3.23
1210	3.79
1450	3.59
2390	4.75
2620	4.92
2875	5.59
3835	6.40
4065	6.77
4310	6.73
5270	7.43
5500	6.90
5750	6.85

Trial #5

Time (min)	Log cfu/mL
0	3.40
970	3.54
1220	3.61
1420	3.86
2435	5.72
2675	5.48
2935	6.08
3865	6.62
4105	6.51
4405	6.61
5390	7.43
5630	7.43

APPENDIX 8.6. T_{min} values of psychrotrophic pseudomonads (Literature Data)

Source	Author	Conditions	n	r^2	T_{min} (K)	Factor measured
dairy	Chandler & McMeekin (1985a)	uninoculated past hom milk	≈>50	0.95	264.7	time to log 7.5
		Ps. E5.2 in broth	34	≈>0.98	267.6	time to 50% ΔA
	Chandler & McMeekin (1985b)	uninoculated raw milk	25	0.988	264.7	time to log 7.5
	Foster *et al* (1958)	uninoculated raw milk	3	0.923	268.8	shelf life
	Fu *et al* (1991)	*P.fragi* ATCC 4973 in milk	7	0.998	265.2	growth rate (vc)
			7	0.993	265.4	length of lag (vc)
	Greene & Jezeski (1954)	*P. fluorescens* #92	4	0.994	268.4	growth rate (vc)
		P. fluorescens #69		0.989	268.5	
	Griffiths & Phillips (1988b)	Uninoculated past milk	46	>0.95	265.8	Av of Tmin from (vc) growth rate, length of lag &time to log 7.5
	Hankin *et al* (1977)	Ps. in uninoculated milk	54	0.96	269.9	time to flavour score <36 (shelf life)
	Ingraham & Stokes (1959)	*P. fluroescens*	4	0.989	267.7	growth rate (vc)
	Langeveld & Cuperus (1980)	Ps. 423a in milk	10	0.993	265.3	growth rate (vc)
	Macario (*pers comm*)	*P.fluorescens* in broth	125	0.957	264.9	growth rate (%T)
		P.fluorescens in whole milk	6	0.941	262.0	growth rate (vc)
		P.fluorescens in modified milk	6	0.993	266.8	growth rate (vc)

Source	Author	Conditions	n	r^2	T_{min} (K)	Factor measured
Dairy	Maxcy & Liewen (1989)	psychrotrophs in broth	6	0.997	266.3	growth rate (vc)
(cont)	Neumeyer (this study)	uninoculated raw milk	13	0.966	265.2	growth rate (vc)
		P.putida 1442 in broth	56	0.996	265.3	growth rate (%T)
		P.putida 1442 in modified milk	34	0.995	265.2	growth rate (vc)
		P.putida 1442 in whole milk	21	0.981	265.5	growth rate (vc)
		Ps. cocktail (5 strains) in broth	58	0.987	264.7	growth rate (%T, av2))
		P.fluorescens 1412 in broth	30	0.993	264.9	growth rate (%T)
		P.fragi I6 in broth	90	0.992	267.3	growth rate (%T, av3)
		P.fragi ncimb 8542 in broth	56	0.997	266.0	growth rate (%T,av2)
	Phillips & Griffiths (1987)	*P.fluorescens* 1181 in milk products	16	≈>0.99	269.7	time for 4 logs growth;
		P.fluorescens 1588 in milk products	16	≈>0.99	262.0	each one av of 4 expts
		P.fragi K1/22 in milk products	16	≈>0.99	261.7	
		P.putida 1587 in milk products	16	≈>0.99	263.4	
		P.stutzeri B4/4 in milk products	16	≈>0.99	262.3	
	Robinson (1981)	Uninoculated raw milk	4	0.968	268.5	growth rate (vc)
	Shelley *et al* (1986)	*P.fluorescens* AS7c1 in UHT milk	6	0.982	264.3	growth rate (vc)
		P.fluorescens AS11a1 in UHT milk	6	0.987	268.2	
		P.fluorescens AS31a1 in UHT milk	6	0.939	263.3	
		P.fragi AS24b1 in UHT milk	6	0.898	256.1	

Source	Author	Conditions	n	r^2	T_{min} (K)	Factor measured
dairy (cont)	Tatini *et al* (1991)	Uninoculated raw milk	84	1.0	265.1	growth rate (vc)
flesh	Ayres (1960)	Ps. on uninoculated beef	5	0.986	264.6	growth rate (vc)
	Cain & Powell (1986)	shelf life of meat	5	0.999	266.9	shelf life(?vc)
	Coates (*pers comm*)	Ps on meat	5	0.865	266.2	growth rate (vc)
	Delaquis & McCurdy (1990)	*P.fluorescens* CC-840406-E on beef	2	1.0	268.8	growth rate (vc)
		P.fragi D5 on beef	2	1.0	267.7	
	Gill & Newton (1977)	non-fluorescent Ps.on meat slices	4	0.995	261.4	growth rate (vc)
		fluorescent Ps. on meat slices	4	0.999	262.4	
	Ingraham (1958)	Ps. 21-3c in broth	17	0.990	265.8	growth rate (%T)
		Ps. 1-3b	16	0.982	260.8	
	Kamperman (1994)	Ps. v4 in broth	33	0.997	266.7	all growth rate (%T)
		Ps. 5 in broth	30	0.994	265.4	
		Ps. 3 in broth	89	>0.991	266.3	av of 3 expts
		Ps. cocktail (5 strains) in broth	60	>0.988	265.2	av of 2 expts
	Muermans *et al* (1993)	Ps. on beef	4	0.971	262.3	growth rate (vc)
	Pooni & Mead (1984)	pigmented Ps. sp. in broth	6	0.996	265.8	growth rates (?vc or
		non-pigmented Ps. sp in broth	6	0.987	266.7	%T)

Source	Author	Conditions	n	r^2	T_{min} (K)	Factor measured
flesh (cont)	Ratkowsky *et al* (1982)	Ps. Group I strain 16L16 in broth	188	0.991	264.0	growth rate (%T)
		Ps. Group III strain G268 in broth	31	0.979	272.1	
		Ps. Group IV strain G489 in broth	74	0.991	263.1	
	Ratkowsky et al (1983)	Ps. Grp I strain 6.4 in broth	16	*	267	growth rate (%T)
		Ps. Grp I strain 4.54 in broth	16		272	
		Ps. Grp II strain 5.16 in broth	16		266	
		Ps. Grp II strain 6 in broth	16		269	
	Scott (1937)	Ps. on beef	8	0.990	266.1	growth rates (vc)
			8	0.986	259.3	
			8	0.992	261.2	
			7	0.983	262.3	
	Stannard *et al* (1985)	non-pigmented Ps. EBT 2/167 in broth	6	0.989	265.2	time to increase 2 log
		pigmented Ps. MJT/F4/14 in broth	6	0.978	265.2	cycles (vc)

past = pasteurised
hom = homogenised
Ps. = pseudomonads
vc = viable count
%T = %Transmittance

av = average eg, av2 = average of 2 experiments
expts = experiments
n = number of data points in the square root plot
r^2 = regression coefficient^2
* = unknown